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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PO 9263 for a patent by THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH filed on 17 September 1997.

I further certify that the annexed specification is not, as yet, open to public inspection.

WITNESS my hand this Twenty-eighth day of September 1998

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Regulation 3.2

The Walter and Eliza Hall Institute of Medical Research

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Novel Therapeutic Molecules"

The invention is described in the following statement:

NOVEL THERAPEUTIC MOLECULES

The present invention relates generally to novel molecules capable of, *inter alia*, modulating apoptosis in mammalian cells and to genetic sequences encoding same. More particularly, the present invention relates to a novel member of the Bcl-2 family of proteins, referred to herein as "Bim", and to genetic sequences encoding same. The molecules of the present invention are useful, for example, in therapy, diagnosis, antibody generation and as a screening tool for therapeutic agents capable of modulating physiological cell death or survival and/or modulating cell cycle entry.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography. A summary of the SEQ ID NOs. is provided before the Examples.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Apoptosis, the physiologic and genetically modulated process of cell death, is of central importance for modelling tissues and maintaining homeostasis in multicellular organisms (Kerr *et al.*, 1972; Jacobson *et al.*, 1997). Great progress is being made towards understanding the biochemistry underlying this intrinsic suicide program. The cellular apoptotic effector molecules include a set of cysteine proteinases, termed caspases, that degrade critical cellular substrates (Nicholson and Thornberry, 1997). The regulatory machinery that governs the activation of the caspases is less well understood. However a family of proteins of which Bcl-2 is the prototypic molecule (and is referred to as the Bcl-2 family of proteins) plays a central role (Jacobson, 1997; Reed, 1997; Kroemer, 1997).

Bcl-2 was the first intracellular regulator of apoptosis to be identified (Vaux *et al.*, 1988) and high levels enhance cell survival under diverse cytotoxic conditions. Other cellular homologs, such as Bcl-x_L (Boise *et al.*, 1993) and Bcl-w (Gibson *et al.*, 1996), also enhance cell survival, as do more distantly related viral homologs, such as the adenovirus E1B 19K protein (White *et al.*, 1992) and Epstein-Barr virus BHRF-1 (Henderson *et al.*, 1993). However, the family also includes members such as Bax (Oltvai *et al.*, 1993) and Bak (Chittenden *et al.*, 1995; Kiefer *et al.*, 1995; Farrow *et al.*, 1995), which antagonise the activity of the pro-survival proteins and provoke apoptosis when expressed at high concentrations. The relative concentrations of the opposing sub-family members may determine whether the cell lives or dies (Oltvai *et al.*, 1993).

The homology between members of the Bcl-2 family is greatest within four small regions, designated Bcl-2 Homology (BH) regions (Yin *et al.*, 1994; Borner *et al.*, 1994; Chittenden *et al.*, 1995; Gibson *et al.*, 1996; Zha *et al.*, 1996). The N-terminal BH4 domain is restricted to some antagonists of apoptosis, while BH1, BH2 and BH3 can be found in both sub-families (reviewed by Kroemer, 1997). In the tertiary structure determined for Bcl-x_L, the BH1, BH2 and BH3 domains form an elongated hydrophobic cleft on the surface of the molecule, stabilised by the BH4 amphipathic helix (Muchmore *et al.*, 1996; Sattler *et al.*, 1997). Most members of the Bcl-2 family contain a C-terminal hydrophobic region, which appears to be important for their localisation to intracytoplasmic membranes (reviewed by Kroemer, 1997).

Protein interactions are an important feature of the Bcl-2 family. Interaction between the pro-survival and pro-apoptotic proteins, such as Bcl-2 with Bax or Bak, requires the BH1 and BH2 domains of the former (Yin *et al.*, 1994; Sedlak *et al.*, 1995; Hanada *et al.*, 1995) and the BH3 domain of the latter (Chittenden *et al.*, 1995; Zha *et al.*, 1996). BH3 peptides bind to the hydrophobic cleft of Bcl-x_L formed by BH1, BH2 and BH3 (Sattler *et al.*, 1997). Although mutagenesis of Bcl-2 and Bcl-x_L initially suggested that their ability to inhibit cell death required binding to a pro-apoptotic family member (Yin *et al.*, 1994), Bcl-x_L mutants have been identified that do not bind Bax or Bak but still block apoptosis (Cheng *et al.*, 1996).

An additional group of pro-apoptotic proteins has recently been described – Bik/Nbk (Boyd *et al.*, 1995; Zha *et al.*, 1996), Bid (Wang *et al.*, 1996) and Hrk (Inohara *et al.*, 1997). The only feature they share in common with each other, or the Bcl-2 family, is the small (9 amino acid) BH3 domain. This region is essential for the ability of these proteins to promote cell death.

In work leading up to the present invention, the inventors have identified a novel member of the Bcl-2 family, designated herein "Bim". In accordance with the present invention, Bim induces cell death and acts as a "death-ligand" for certain members of the pro-survival Bcl-2 family. The identification of this new gene permits the identification and rational design of a range of products for use in therapy, diagnosis, antibody generation and involving modulation of physiological cell death. These therapeutic molecules may act as either antagonists or agonists of Bim expression or activity and will be useful in cancer or degenerative disease therapy.

Accordingly, one aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in one of SEQ ID NO: 2, 4, or 6 or a derivative or homolog thereof or having at least about 45% or greater similarity to one or more of SEQ ID NO: 2, 4, or 6, or a derivative or homolog thereof.

Another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in one of SEQ ID NO: 8 or 10 or a derivative or homolog thereof or having at least about 45% or greater similarity to one or more of SEQ ID NO: 8 or 10 or a derivative or homolog thereof.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in

different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels.

Another aspect of the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in one of SEQ ID NO: 1, 3, or 5 or a derivative or homolog thereof capable of hybridising to one of SEQ ID NO: 1, 3, or 5 under low stringency conditions at 42°C and which encodes an amino acid sequence corresponding to an amino acid sequence set forth in one of SEQ ID NO: 2, 4 or 6 or a sequence having at least about 45% similarity to one or more of SEQ ID NO: 2, 4, or 6.

More particularly the present invention contemplates a nucleic acid molecule comprising a sequence of nucleotides substantially as set forth in SEQ ID NO: 1, 3, or 5.

Another aspect of the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in one of SEQ ID NO: 7 or 9 or a derivative of homolog thereof capable of hybridising to one of SEQ ID NO: 7 or 9 under low stringency conditions at 42°C and which encodes an amino acid sequence corresponding to an amino acid sequence set forth in one of SEQ ID NO: 8 or 10 or a sequence having at least about 45% similarity to one or more of SEQ ID NO: 8 or 10.

More particularly the present invention contemplates a nucleic acid molecule comprising a sequence of nucleotides substantially as set forth in SEQ ID NO: 7 or 9.

Reference herein to a low stringency at 42°C includes and encompasses from at least about 1% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for

washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions.

The nucleic acid molecule according to this aspect of the present invention corresponds herein to "*Bim*". This gene has been determined in accordance with the present invention to induce apoptosis. The product of the *Bim* gene is referred to herein as "Bim". Bim is known as a "BH3-only" protein since the only Bcl-2 homology region which it expresses is BH3. It thereby forms a novel member of a Bcl-2 related BH3-only pro-apoptotic group which also comprises, for example, Bik/Nbk, Bid and Hrk. However, Bim is the only BH3-only protein for which splice variants exist, thereby resulting in the expression of a variety of isoforms. Bim_S, Bim_L and Bim_{EL} are examples of three said isoforms which differ in both size and potency of functional activity. Murine Bim $_S$, Bim $_L$ and Bim $_{EL}$ are defined by the amino acid sequences set forth in SEQ ID NO: 2, 4 and 6, respectively and human Bim_L and Bim_{EL} are defined by the amino acid sequences set forth in SEQ ID NO: 8 and 10, respectively. The cDNA nucleotide sequences for murine Bim_S, Bim_L and Bim_{EL} are defined by the nucleotide sequences set forth in SEQ ID NO: 1, 3 and 5, respectively and human Bim_L and Bim_{EL} are defined by the nucleotide sequences set forth in SEQ ID NO: 7 and 9, respectively.

The nucleic acid molecule encoding *Bim* is preferably a sequence of deoxyribonucleic acids such as cDNA sequence or a genomic sequence. A genomic sequence may also comprise exons and introns. A genomic sequence may also include a promoter region or other regulatory region.

Reference hereinafter to "Bim" and "*Bim*" should be understood as a reference to all forms of Bim and *Bim*, respectively, including, by way of example, the three peptide and cDNA isoforms of Bim_S, Bim_L and Bim_{EL} which have been identified as arising from alternative splicing of mRNA. Reference hereinafter to Bim and *Bim* includes reference to derivatives thereof.

The protein and/or gene is preferably from a human, primate, livestock animal (eg. sheep, pig, cow, horse, donkey) laboratory test animal (eg. mouse, rat, rabbit, guinea pig) companion animal (eg. dog, cat), captive wild animal (eg. fox, kangaroo, deer), aves (eg. chicken, geese, duck, emu, ostrich), reptile or fish.

Derivatives include fragments, parts, portions, chemical equivalents, mutants, homologs from natural, synthetic or recombinant sources including fusion proteins. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences including fusions with other peptides, polypeptides or proteins.

The derivatives of Bim include fragments having particular epitopes of parts of the entire Bim protein fused to peptides, polypeptides or other proteins. Analogs of Bim contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecule or their analogs.

Derivatives of nucleic acid sequences may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules of the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in cosuppression and fusion of nucleic acid molecules.

The nucleic acid molecule of the present invention is preferably in isolated form or ligated to a vector, such as an expression vector. By "isolated" is meant a nucleic acid molecule having undergone at least one purification step and this is conveniently defined, for example, by a composition comprising at least about 10% subject nucleic acid molecule, preferably at least about 20%, more preferably at least about 30%, still more preferably at least about 40-50%, even still more preferably at least about 60-70%, yet even still more preferably 80-90% or greater of subject nucleic acid molecule relative to other components as determined by molecular weight, encoding activity, nucleotide sequence, base composition or other convenient means. The nucleic acid molecule of the present invention may also be considered, in a preferred embodiment, to be biologically pure.

In a particularly preferred embodiment, the nucleotide sequence corresponding to *Bim* is a cDNA sequence comprising a sequence of nucleotides as set forth in one of SEQ ID NO: 1, 3 or 5 or is a derivative or homolog thereof including a nucleotide sequence having similarity to one of SEQ ID NO: 1, 3 or 5 and which encodes an amino acid sequence corresponding to an amino acid sequence as set forth in one of SEQ ID NO: 2, 4 or 6 or a sequence having at least about 45% similarity to one or more of SEQ ID NO: 2, 4, or 6.

In another particularly preferred embodiment, the nucleotide sequence corresponding to *Bim* is a cDNA sequence comprising a sequence of nucleotides as set forth in one of SEQ ID NO: 7 or 9 or is a derivative or homolog thereof including a nucleotide sequence having similarity to one of SEQ ID NO: 7 or 9 and which encodes an amino

acid sequence corresponding to an amino acid sequence as set forth in one of SEQ ID NO: 8 or 10 or a sequence having at least about 45% similarity to one or more of SEQ ID NO: 8 or 10.

A derivative of the nucleic acid molecule of the present invention also includes nucleic acid molecules capable of hybridising to the nucleotide sequences as set forth in one of SEQ ID NO: 1, 3, or 5 or SEQ ID NO: 7 or 9 under low stringency conditions. Preferably, said low stringency is at 42°C.

In another embodiment the present invention is directed to an isolated nucleic acid molecule encoding *Bim* or a derivative thereof, said nucleic acid molecule selected from the list consisting of:

(i) A nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence set forth in one of SEQ ID NO: 2, 4, or 6 or a derivative or homolog thereof or having at least about 45%similarity to one or more of SEQ ID NO: 2, 4, or 6.

(ii) A nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence set forth in one of SEQ ID NO: 8 or 10 or a derivative or homolog or having at least about 45% similarity to one of SEQ ID NO: 8 or 10.

(iii) A nucleic acid molecule comprising a nucleotide sequence substantially as set forth in one of SEQ ID NO: 1, 3, or 5 or a derivative or homolog thereof.

(iv) A nucleic acid molecule comprising a nucleotide sequence substantially as set forth in one of SEQ ID NO: 7 or 9 or a derivatice or homolog thereof.

(v) A nucleic acid molecule capable of hybridising under low stringency conditions at 42°C to the nucleotide sequence substantially as set forth in one of SEQ ID NO: 1, 3, or 5 a derivative or homolog and encoding an amino acid sequence

corresponding to an amino acid sequence as set forth in one of SEQ ID NO: 2, 4 or 6 a derivative or homolog or a sequence having at least about 45% similarity to one or more of SEQ ID NO: 2, 4, or 6.

(vi) A nucleic acid molecule capable of hybridising under low stringency conditions at 42°C to the nucleotide sequence substantially as set forth in one of SEQ ID NO: 7 or 9 a derivative or homolog and encoding an amino acid sequence corresponding to an amino acid sequence as set forth in one of SEQ ID NO: 8 or 10 a derivative or homolog or a sequence having at least about 45% similarity to one or more of SEQ ID NO: 8 or 10.

(vii) A nucleic acid molecule capable of hybridising to the nucleic acid molecule of paragraphs (i) or (iii) or (v) under low stringency conditions at 42°C and encoding an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO: 2, 4, or 6.

(viii) A nucleic acid molecule capable of hybridising to the nucleic acid molecule of paragraphs (ii) or (iv) or (vi) under low stringency conditions at 42°C and encoding an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO: 8 or 10.

(ix) A derivative or mammalian homolog of the nucleic acid molecule of paragraphs (i) or (ii) or (iii) or (iv) or (v) or (vi) or (vii) or (viii).

The nucleic acid molecule may be ligated to an expression vector capable of expression in a prokaryotic cell (e.g. *E.coli*) or a eukaryotic cell (e.g. yeast cells, fungal cells, insect cells, mammalian cells or plant cells). The nucleic acid molecule may be ligated or fused or otherwise associated with a nucleic acid molecule encoding another entity such as, for example, a signal peptide, a cytokine or other member of the Bcl-2 family.

The present invention extends to the expression product of the nucleic acid molecule hereinbefore defined.

The expression product is Bim having an amino acid sequence set forth in one of SEQ ID NO: 2, 4, 6, 8 or 10 or is a derivative or homolog thereof as defined above or is a mammalian homolog having an amino acid sequence of at least about 45% similarity to the amino acid sequence set forth in one of SEQ ID NO: 2, 4, 6, 8 or 10 or derivative or homolog thereof.

Another aspect of the present invention is directed to an isolated polypeptide selected from the list consisting of:

(i) A polypeptide having an amino acid sequence substantially as set forth in one of SEQ ID NO: 2, 4, or 6 or derivative or homolog thereof or a sequence having at least about 45% similarity to one or more of SEQ ID NO: 2, 4, or 6.

(ii) A polypeptide having an amino acid sequence substantially as set forth in one of SEQ ID NO: 8 or 10 a derivative or homolog or a sequence having at least about 45% similarity to one or more of SEQ ID NO: 8 or 10.

(iii) A polypeptide encoded by a nucleotide sequence substantially as set forth in one of SEQ ID NO: 1, 3, or 5 or derivative or homolog thereof or a sequence encoding an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO: 2, 4, or 6.

(iv) A polypeptide encoded by a nucleotide sequence substantially as set forth in one of SEQ ID NO: 7 or 9 or derivative or homolog thereof or a sequence encoding an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO: 8 or 10.

(v) A polypeptide encoded by a nucleic acid molecule capable of hybridising to the nucleotide sequence as set forth in one of SEQ ID NO: 1, 3, or 5 or derivative or homolog thereof under low stringency conditions at 42°C and which encodes an amino acid sequence substantially as set forth in SEQ ID NO: 2, 4, or 6 or derivative or homolog thereof or an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO: 2, 4, or 6.

(vi) A polypeptide encoded by a nucleic acid molecule capable of hybridising to the nucleotide sequence as set forth in one of SEQ ID NO: 7 or 9 or derivative or homolog thereof under low stringency conditions at 42°C and which encodes an amino acid sequence substantially as set forth in SEQ ID NO: 8 or 10 or derivative or homolog thereof or an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO: 8 or 10.

(vii) A polypeptide as defined in paragraphs (i) or (ii) or (iii) or (iv) or (v) or (vi) in homodimeric form.

(viii) A polypeptide as defined in paragraphs (i) or (ii) or (iii) or (iv) or (v) or (vi) in heterodimeric form.

Although not intending to limit the invention to any one theory or mode of action, the BH3 region is responsible for some of the cytotoxic actions of Bim. The BH3 region forms an amphiphathic helix that interacts with the elongated hydrophobic cleft formed by the BH1, BH2 and BH3 regions of pro-survival molecules such as, for example, Bcl-x_L. The pro-apoptotic action of Bim reflects its ability to chelate the anti-apoptotic members of the Bcl-2 family. Bim is the only BH3-only protein for which spliced variants have been described. Isoforms such as Bim_S, Bim_L and Bim_{EL} interact *in vivo* with Bcl-2 family members but induce cell death with different degrees of cytotoxicity. Bim_S, for example, is a more potent inducer of cell death than Bim_L or Bim_{EL}.

The Bim of the present invention may be in multimeric form meaning that two or more molecules are associated together. Where the same Bim molecules are associated together, the complex is a homomultimer. An example of a homomultimer is a homodimer. Where at least one Bim is associated with at least one non-Bim molecule, then the complex is a heteromultimer such as a heterodimer. A heteromultimer may include a molecule of another member of the Bcl-2 family or other molecule capable of modulating apoptosis.

The present invention contemplates, therefore, a method for modulating expression of *Bim* in a mammal, said method comprising contacting the *Bim* gene with an effective amount of an agent for a time and under conditions sufficient to up-regulate or down-regulate or otherwise modulate expression of *Bim*. For example, *Bim* antisense sequences such as oligonucleotides may be introduced into a cell to enhance the ability of that cell to survive. Conversely, a nucleic acid molecule envoding Bim or a derivative thereof may be introduced to decrease the survival capacity of any cell expressing the endogenous *Bim* gene.

Another aspect of the present invention contemplates a method of modulating activity of Bim in a mammal, said method comprising administering to said mammal a modulating effective amount of an agent for a time and under conditions sufficient to increase or decrease Bim activity.

Modulation of said activity by the administration of an agent to a mammal can be achieved by one of several techniques, including but in no way limited to introducing into said mammal a proteinaceous or non-proteinaceous molecule which:

(i) modulates expression of *Bim*;

(ii) functions as an antagonist of Bim;

(iii) functions as an agonist of Bim.

Said proteinaceous molecule may be derived from natural or recombinant sources including fusion proteins or following, for example, natural product screening. Said non-proteinaceous molecule may be, for example, a nucleic acid molecule or may be derived from natural sources, such as for example natural product screening or may be chemically synthesised. The present invention contemplates chemical analogs of Bim capable of acting as agonists or antagonists of Bim. Chemical agonists may not necessarily be derived from Bim but may share certain conformational similarities. Alternatively, chemical agonists may be specifically designed to mimic certain physiochemical properties of Bim. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing Bim from carrying out their normal biological functions. Antagonists include monoclonal antibodies specific for Bim, or parts of Bim, and antisense nucleic acids which prevent transcription or translation of *Bim* genes or mRNA in mammalian cells.

Increased *Bim* expression or Bim activity may be important, for example, for treatment or prophylaxis in conditions such as cancer. Decreased *Bim* expression or Bim activity may be influential in regulating inhibition or prevention of cell degeneracy such as under cytotoxic conditions during, for example, γ-irradiation and chemotherapy.

Another aspect of the present invention contemplates a method of modulating apoptosis in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a nucleotide sequence encoding *Bim*.

Yet another aspect of the present invention contemplates a method of modulating apoptosis in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of Bim.

Still another aspect of the present invention contemplates a method of modulating apoptosis in a mammal said method comprising administering to said mammal an effective amount of Bim or *Bim*.

The Bim, *Bim* or agent used may also be linked to a targeting means such as a monoclonal antibody, which provides specific delivery of the Bim, *Bim* or agent to the target cells.

In a preferred embodiment of the present invention, the Bim, *Bim* or agent used in the method is linked to an antibody specific for said target cells to enable specific delivery to these cells.

Administration of the Bim, *Bim* or agent, in the form of a pharmaceutical composition, may be performed by any convenient means. Bim, *Bim* or agent of the pharmaceutical composition are contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the Bim, *Bim* or agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of Bim or agent may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation. The Bim or agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). With particular reference to use of Bim or agent, these peptides may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

A further aspect of the present invention relates to the use of the invention in relation to mammalian disease conditions. For example, the present invention is particularly useful, but in no way limited to, use in cancer therapy.

Accordingly, another aspect of the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of *Bim* wherein said modulation results in modulation of apoptosis.

In another aspect the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of Bim wherein said modulation results in modulation of apoptosis.

In another aspect the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of Bim for a time and under conditions sufficient to modulate apoptosis.

Yet another aspect the present invention relates to a method of treating a mammal said method comprising administering to said mammal an effective amount of *Bim* for a time and under conditions sufficient to modulate apoptosis.

In yet another aspect the present invention relates to the use of an agent capable of modulating the expression of *Bim* in the manufacture of a medicament for the modulation of apoptosis.

Another aspect of the present invention relates to the use of an agent capable of modulating the expression of Bim in the manufacture of a medicament for the moduclation of apoptosis.

A further aspect of the present invention relates to the use of Bim or *Bim* in the manufacture of a medicament for the moduclation of apoptosis.

Still yet another aspect of the present invention relates to agents for use in modulating *Bim* expression wherein modulating expression of said *Bim* modulates apoptosis.

A further aspect of the present invention relates to agents for use in modulating Bim expression wherein modulating expression of said Bim modulates apoptosis.

Another aspect of the present invention relates to Bim or *Bim* for use in modulating apoptosis.

In a related aspect of the present invention, the mammal undergoing treatment may be human or an animal in need of therapeutic of prophylactic treatment.

In yet another further aspect the present invention contemplates a pharmaceutical composition comprising *Bim*, Bim or an agent capable of modulating *Bim* expression or Bim activity together with one or more pharmaceutically acceptable carriers and/or diluents. *Bim*, Bim or said agent are referred to as the active ingredients.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as licithin, by the maintenance of the required particle size in the

case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When *Bim,* Bim and Bim modulators are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained.

Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 ug and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein

refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5 μg to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.5 μg to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of modulating *Bim* expression or Bim activity. The vector may, for example, be a viral vector.

Conditions requiring modulation of physiological cell death include enhancing survival of cells utilising, for example, antisense sequence in patients with neurodegenerative diseases, myocardial infarction, muscular degenerative disease, hypoxia, ischaemia, HIV infection or for prolonging the survival of cells being transplanted for treatment of disease. Alternatively, the molecules of the present invention are useful for, for example, reducing the survival capacity of tumour cells or autoreactive lymphocytes.

The anti-sense sequence may also be used for modifying *in vitro* behaviour of cells, for example, as part of a protocol to develop novel lines from cell types having unidentified growth factor requirements; for facilitating isolation of hybridoma cells producing monoclonal antibodies, as described below; and for enhancing survival of cells from primary explants while they are being genetically modified.

Still another aspect of the present invention is directed to antibodies to Bim including catalytic antibodies. Such antibodies may be monoclonal or polyclonal and may be selected from naturally occurring antibodies to Bim or may be specifically raised to Bim. In the case of the latter, Bim may first need to be associated with a carrier molecule. The antibodies and/or recombinant Bim of the present invention are particularly useful as therapeutic or diagnostic agents. Alternatively, fragments of antibodies may be used such as Fab fragments. Furthermore, the present invention extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies. The antibodies of this aspect of the present invention are particularly useful for immunotherapy and may also be used as a diagnostic tool for assessing apoptosis or monitoring the program of a therapeutic regime.

For example, Bim can be used to screen for naturally occurring antibodies to Bim. These may occur, for example in some autoimmune diseases.

For example, specific antibodies can be used to screen for Bim proteins. The latter would be important, for example, as a means for screening for levels of Bim in a cell extract or other biological fluid or purifying Bim made by recombinant means from culture supernatant fluid. Techniques for the assays contemplated herein are known in the art and include, for example, sandwich assays, ELISA and flow cytometry.

It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal or fragments of antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first

antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of Bim.

Both polyclonal and monoclonal antibodies are obtainable by immunization with the protein or peptide derivatives and either type is utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of Bim, or antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of the product.

The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example Douillard and Hoffman, Basic Facts about Hybridomas, in *Compendium of Immunology* Vol II, ed. by Schwartz, 1981; Kohler and Milstein, *Nature 256*: 495-499, 1975; *European Journal of Immunology 6*: 511-519, 1976).

Another aspect of the present invention contemplates a method for detecting Bim in a biological sample from a subject said method comprising contacting said biological sample with an antibody specific for Bim or its derivatives or homologs for a time and under conditions sufficient for an antibody-Bim complex to form, and then detecting said complex.

The presence of Bim may be determined in a number of ways such as by Western blotting, ELISA or flow cytometry procedures. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labelled antibody

to a target.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention the sample is one which might contain Bim including cell extract, tissue biopsy or possibly serum, saliva, mucosal secretions, lymph, tissue fluid and respiratory fluid. The sample is, therefore, generally a biological sample comprising biological fluid but also extends to fermentation fluid and supernatant fluid such as from a cell culture.

In the typical forward sandwich assay, a first antibody having specificity for the Bim or antigenic parts thereof, is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the

sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes) and under suitable conditions (e.g. 25°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the hapten. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the hapten.

An alternative method involves immobilizing the target molecules in the biological sample and then exposing the immobilized target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the

chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorecein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescene and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

The present invention also contemplates genetic assays such as involving PCR analysis to detect *Bim* or its derivatives.

Further features of the present invention are more fully described in the following examples. It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention. It should not be understood in any way as a restriction on the broad description of the invention as set out above.

Figure 1 is a schematic representation of the isolation of cDNAs encoding three isoforms of Bim. (A) Open reading frames of five independent clones isolated by screening a cDNA expression library with recombinant Bcl-2 protein. Dotted lines indicate putative splices and arrows indicate PCR primers spanning the splice sites. (B) Relationship of the three Bim isoforms. The black box denotes the BH3 homology region and the hatched box the predicted hydrophobic region. Regions specific to the larger splice variants are shaded. (C) Sequence alignment of the mouse and human Bim_{EL} polypeptide sequences using the GCG "BESTFIT" program; identical residues appear on a dark background. The BH3 homology region and the C-terminal hydrophobic region predicted by the Kyte-Doolittle algorithm are boxed. Arrows indicate residues present only in the longer isoforms. Since the nucleotide sequences of the mouse and human cDNAs diverged 5' of the predicted initiating ATG and there are stop codons in all three reading frames upstream of the human open reading frame, that start codon is likely to be correct.

Figure 2 is a photographic representation of the expression of *bim* RNA in hematopoietic cell lines. Northern blot analysis of polyA$^+$ RNA, using a mouse *bim* cDNA probe. The RNAs were derived from the following mouse lines: T lymphomas KO52DA20 (lanes 1 to 5), WEHI 703 (lane 6), WEHI 707 (lane 7) and WEHI 7.1 (lane 8); B lymphomas CH1 (lanes 9, 10) and WEHI 231 (lanes 11, 12); pre-B lymphoma WEHI 415 (lane 13); T hybridoma B6.2.16 BW2 (lanes 14, 15); myeloid progenitor FDC-P1 (lane 16). Those lines that harbor a *bcl-2* expression vector or transgene are indicated. Certain RNAs were isolated from cells exposed to cytotoxic conditions: 1 μM dexamethasone (14 hr, lanes 2 and 4; 24 hr, lane 5); γ-irradiation (10 Gy) (lane 5). Samples from a single autoradiograph have been rearranged in order electronically.

Figure 3 is a photographic representation of the localisation of Bim protein to intracellular membranes. (A) L929 fibroblasts transiently transfected with EE-tagged Bim_L were fixed, permeabilised and stained with the anti-EE antibody; fluorescence was visualised by confocal microscopy. (B) and (C) L929 cells stably co-expressing human Bcl-2 and EE-tagged Bim_L were stained with anti-human Bcl-2 antibody (B) or anti-EE antibody (C). (D) Images from the staining with anti-EE (B) and anti-Bcl-2 (C) were

superimposed; co-localisation is indicated by (*colour) staining.

Figure 4 is a graphical representation demonstrating that Bim induces apoptosis and can be inhibited by p35 and Bcl-2 but not CrmA. (A) Flow cytometric DNA analysis (see Materials and Methods) of 293T cells transfected 24 h previously with *EE-bim$_L$* plasmid (0.5 μg). (B) Kinetics of apoptosis elicited by EF-*bim$_L$* plasmid (0.5 μg), assessed as in A. (C) Cell viability 48 h after transfection with 0.1, 0.2 or 0.5 μg of *EE-bim$_L$* plasmid alone (black bars) or together with 0.5 μg of wild-type or mutant *p35* or *crmA* plasmid (grey bars). (D) Cell viability 48 h after transfection with 0.1, 0.2 or 0.5 μg of *EE-bim$_L$* plasmid together with 0.5 μg of the indicated wt or mutant *bcl-2* plasmids. C and D show the percentage of viable Bim-expressing cells, determined by DNA FACS analysis, as in A, and are the mean ± SD of 3 or more independent experiments.

Figure 5 is a graphical representation demonstrating that Bim antagonises the anti-apoptotic activity of Bcl-2 in a dose-dependent fashion. (A) Immunofluorescence staining of cloned FDC-P1 cell lines stably expressing Bcl-2 alone (dashed line) or co-expressing Bcl-2 and varying levels of EE-Bim$_L$ (solid lines). (B) Viability of these clones when cultured in the absence of IL-3 or after exposure to γ-irradiation (10 Gy). Cell viability was assessed by vital dye exclusion; data shown are means ± SD of at least 3 experiments and are representative of results obtained with at least 3 independent lines of each genotype.

Figure 6 is a graphical representation of a comparison of the activity of the three Bim isoforms. (A) Immunofluorescence staining of cloned FDC-P1 lines expressing Bcl-2 alone (dotted) or Bcl-2 plus EE-tagged Bim$_L$, Bim$_{EL}$ or Bim$_S$ (solid lines). (B) Association of EE-tagged Bim$_S$, Bim$_L$ and Bim$_{EL}$ with Bcl-2 demonstrated by anti-EE immunoblots of immunoprecipitates prepared with anti-human Bcl-2 monoclonal antibody from FDC-P1 cells expressing the indicated proteins. The 25 kD protein is non-specific. (C) Effect of Bim isoforms on viability of FDC-P1 cells expressing Bcl-2, after removal of growth factor or exposure to irradiation.

Figure 7 is a graphical representation demonstrating that Bim binds to and antagonises Bcl-x_L or Bcl-w but not E1B19K. (A) Lysates of ^{35}S-labelled 293T cells transiently co-transfected with the plasmids encoding the indicated proteins were immunoprecipitated with anti-EE antibody, and the EE-Bim_L-containing complexes were fractionated by SDS-PAGE. (B) Lysates from parental 293T cells or 293T cells co-expressing EE-tagged Bim_L and FLAG-tagged Bcl-x_L, Bcl-w or E1B19K were immunoblotted directly or after immunoprecipitation, as indicated. (C, D) 293T cells were transiently transfected with a vector control (unfilled bar) or with 0.1, 0.2 or 0.5 μg of EE-Bim_L plasmid, either alone (black bars) or together with 0.5 μg of plasmids encoding wt or mutant Bcl-x_L (C); Bcl-w or E1B19K protein (D) (grey bars). The flow cytometric analysis was as described in the legend to Figure 4. Data shown are means ± SD of 3 or more independent experiments.

Figure 8 is a graphical representation demonstrating that the BH3 homology region of Bim is required for binding to and inhibiting Bcl-2. (A) Immunofluorescence staining of cloned FDC-P1 lines expressing Bcl-2 alone (dotted) or with EE-Bim_L or EE-Bim ÆBH3 (solid line), and of EE-Bim ÆBH3 in the parental FDC-P1 cells (broken line). (B) Immunoblot showing that Bcl-2 associates with wild-type Bim_L but not the ÆBH3 mutant. (C) Viability of FDC-P1 clones expressing the indicated proteins (see A) was assessed by vital dye exclusion. Data shown are means ± SD of at least 3 experiments and are representative of results obtained with at least 3 independent lines of each genotype.

Figure 9 is a diagrammatic representation of the BH3 homology regions in the Bcl-2 family. (A) Amino acid sequences of the human proteins were aligned with the modified method of Feng and Doolittle used by the GCG "PILEUP" program (Feng and Doolittle, 1987). Residues that are identical or very similar (K & R; D & E; V & I; M & L) in >8 of the 11 proteins are shaded in dark grey, while less conserved residues (present in >5/11 proteins) are shaded in light grey. (B) Short stretch of amino acid homology between Bim and *C. elegans* Ced-4; this region overlaps with the BH3 region of Bim, indicated by the box.

SUMMARY OF SEQ ID NO:

Sequence	SEQ ID NO:
nucleotide sequence of murine Bim_S	1
amino acid sequence of murine Bim_S	2
nucleotide sequence of murine Bim_L	3
amino acid sequence of murine Bim_L	4
nucleotide sequence of murine Bim_{EL}	5
amino acid sequence of murine Bim_{EL}	6
nucleotide sequence of human Bim_L	7
amino acid sequence of human Bim_L	8
nucleotide sequence of human Bim_{EL}	9
amino acid sequence of human Bim_{EL}	10
peptides	11-13
oligonucleotide primers	14-26

EXAMPLE 1

Isolation of a novel gene encoding a Bcl-2-binding protein

In an attempt to identify novel proteins that bind to Bcl-2, we used human Bcl-2 protein, labelled with ^{32}P (Blanar and Rutter, 1992), to screen a bacteriophage λ cDNA expression library constructed from the $p53^{-/-}$ T lymphoma cell line KO52DA2O (Strasser *et al.*, 1994). A screen of 10^6 clones yielded 12 independent clones. Five encoded the same novel protein, which we named Bim, for Bcl-2 interacting mediator of cell death. Sequence analysis of the *bim* cDNAs revealed three variants of the coding region, apparently produced by alternative splicing (Figure 1A). Reverse transcriptase-PCR on mRNA from KO52DA20 cells gave PCR products of the sizes expected for each of these transcripts, which we designated bim_{EL}, bim_L and bim_S, although the last was in low yield (data not shown). The predicted proteins Bim_{EL}, Bim_L and Bim_S comprise 196, 140 and 110 amino acid residues (Figure 1B). Hybridising human embryo and liver cDNA libraries with mouse *bim* cDNA yielded human cDNAs encoding Bim_L and Bim_{EL}. Human Bim_{EL} is a protein of 198 residues, 89% identical to its mouse counterpart (Figure 1C), and human Bim_L (138 residues) is 85% identical to mouse Bim_L.

Bim has no substantial homology with any protein in current databases. However, scrutiny of its sequence (Figure 1C) revealed a stretch of nine amino acids corresponding to a BH3 homology region (Boyd *et al.*, 1995; Chittenden *et al.*, 1995). Apart from this region, the Bim sequence is unrelated to that of any other BH3-containing protein; it contains no other BH region, nor indeed any other known functional motif. The protein does have a C-terminal hydrophobic region (Figure 1C), raising the possibility that it associates with membranes.

Northern blot analysis showed that *bim* was expressed in a number of B and T lymphoid cell lines, although not in the myeloid line FDC-P1 (Figure 2). A major transcript of 5.7 kb and minor transcripts of 3.8, 3.0, and 1.4 kb were detected.

Neither the level nor relative abundance of these transcripts changed significantly in KO52DA20 cells induced to undergo apoptosis by treatment with dexamethasone (Figure 2, compare lanes 1 and 2, and lanes 3 and 4) or exposure to γ-radiation (compare lanes 1 and 5). Overexpression of *bcl-2* in several of the lines did not affect *bim* mRNA levels (Figure 2).

EXAMPLE 2

Bim localises to cytoplasmic membranes

The presence of the C-terminal hydrophobic domain in Bim prompted us to investigate its subcellular localisation. L929 fibroblasts were transiently transfected with an expression vector encoding Bim_L tagged with an N-terminal EE-epitope, and the permeabilised cells were stained with an anti-EE monoclonal antibody. Confocal microscopy revealed that Bim_L was cytoplasmic and apparently associated with intracellular membranes (Figure 3A). We also introduced the bim_L vector into L929 cells stably infected with a human Bcl-2 encoding retrovirus (Lithgow *et al.*, 1994). The similarity of the anti-EE staining pattern of these cells (Figure 3C) to that of those expressing Bim_L alone (Figure 3A) demonstrated that high concentrations of Bcl-2 did not perturb the localisation of Bim_L. The pattern of Bim_L staining was similar to that reported for Bcl-2 (Monaghan *et al.*, 1992; Krajewski *et al.*, 1993; Lithgow *et al.*, 1994), and overlaying the images obtained from the same cells stained with anti-Bcl-2 (Figure 3B) and anti-EE (Figure 3C) antibodies showed that the two proteins co-localised (Figure 3D).

EXAMPLE 3

Overexpression of Bim kills cells by a pathway requiring caspases

Other known 'BH3-only' proteins (Bik/Nbk, Bid and Hrk) provoke apoptosis when highly expressed (Boyd *et al.*, 1995; Han *et al.*, 1996; Wang *et al.*, 1996; Inohara *et al.*, 1997). We therefore tested whether Bim is cytotoxic by transiently transfecting

293T human embryonal kidney cells with a plasmid encoding EE-Bim_L. The viability of the transfected cells was determined subsequently by flow cytometric analysis of permeabilised cells stained with the anti-EE antibody and the DNA-intercalating dye propidium iodide (PI). Whereas almost all untransfected cells or those transfected with an empty vector remained viable after 24 hr, many of those expressing Bim (*i.e.*, EE-antibody positive) contained sub-diploid DNA (Figure 4A). Indeed, by three days, 90% of the cells expressing Bim_L were dead (Figure 4B). The extent of cell death was proportional to the amount of *bim* DNA transfected (black bars, Figure 4C).

The cells expressing Bim appeared to die by apoptosis, as assessed by cell morphology and the generation of sub-diploid DNA (Figure 4A). As expected, the death process required activation of caspases, because co-expression of baculovirus p35, a competitive inhibitor of many types of caspases (Bump *et al.*, 1995), antagonised Bim-induced cell death, whereas an inactive mutant p35 did not (Figure 4C). Since crmA, a potent inhibitor of caspases 1 and 8 (ICE and FLICE) (Orth *et al.*, 1996; Srinivasula *et al.*, 1996) was not effective (Figure 4C), these particular caspases do not appear to play a critical role.

Numerous failed attempts to generate lines that stably express Bim suggested that it is toxic to diverse cell types. Those repeatedly tested include haemopoietic lines (FDC-P1, CH1, Jurkat, SKW6 and B6.2.16BW2), fibroblastoid lines (Rat-1, NIH3T3 and L929) and an epithelial line (293). The cells were electroporated with a vector encoding puromycin resistance and either EE- or FLAG-tagged Bim_L and selected in puromycin, but no line expressing Bim emerged. A vector encoding untagged Bim also failed to generate viable clones. We quantified the cytotoxicity of Bim by colony assays on transfected L929 fibroblasts. The EE-Bim_L vector yielded only one fifth as many puromycin-resistant colonies as the control vector, and when six of the EE-Bim_L-transfected, drug-resistant colonies were expanded, only one contained any Bim and the level was very low (Table 1 and data not shown). Thus, high levels of Bim suppress clonogenicity and appear incompatible with prolonged cell viability.

EXAMPLE 4

Bim cytotoxicity can be abrogated by wild-type Bcl-2 but not inactive mutants

Co-expression experiments established that Bcl-2 could block cell death induced by Bim_L (Figure 4D). In 293T cells transiently transfected with both the *bcl*-2 and bim_L plasmids, relatively few cells died, even with a high concentration of bim_L DNA (compare the 4th sample in Figure 4C with the 3rd in Figure 4D). The cytotoxicity of *bim*, however, could not be countered by mutant forms of *bcl*-2 rendered inactive by deletion of the BH4 homology region (ΔBH4) (Borner *et al.*, 1994; Huang *et al.*, 1997), or by a point mutation in its BH1 (G145E) or BH2 (W188A) region (Yin *et al.*, 1994) (Figure 4D). Thus, ability to antagonise Bim-induced cell death required a functional Bcl-2 molecule.

High levels of Bcl-2 allowed stable expression of Bim_L. Indeed, when L929 cells stably expressing Bcl-2 were transfected with the EE-Bim_L vector, the frequency of puromycin-resistant colonies approached that obtained with the control vector, and four of six colonies analysed contained moderate to high levels of Bim (Table 1 and data not shown). Similarly, using FDC-P1 clones expressing wt Bcl-2 (but not mutant Bcl-2), we could readily establish sub-clones expressing varying levels of Bim_L (Figure 5A). When grown in the presence of IL-3, all were indistinguishable in growth characteristics and morphology from the parental FDC-P1 cells or those bearing Bcl-2 alone. However, when deprived of IL-3 or irradiated, cells expressing Bcl-2 and a moderate or high level of Bim died more readily than those expressing Bcl-2 alone (Figure 5B). Since each clone had the same level of Bcl-2 (not shown), their sensitivity to apoptosis presumably reflects the ratio of the pro-apoptotic Bim to the anti-apoptotic Bcl-2.

EXAMPLE 5

The three isoforms of Bim all interact with Bcl-2 in vivo but vary in cytotoxicity

We next explored whether all isoforms of Bim were equivalent. An FDC-P1 clone expressing human Bcl-2 was transfected with vectors expressing Bim_{EL}, Bim_L or Bim_S, and puromycin-resistant clones that expressed the same amount of each isoform were selected for further analysis (Figure 6A). To test for association with Bcl-2, immunoprecipitates prepared from cell lysates using a monoclonal antibody specific for human Bcl-2 were fractionated electrophoretically and blotted with anti-EE antibody. Each of the Bim isoforms clearly bound to Bcl-2 (Figure 6B). However, when the transfectants were deprived of IL-3 or subjected to γ-irradiation, it became evident that Bim_S antagonised Bcl-2 more effectively than Bim_L while Bim_{EL} was the least potent (Figures 6C) . In addition, Bim_S suppressed L929 colony formation more effectively than Bim_L or Bim_{EL} (Table 1). Thus, although all three Bim isoforms can bind to Bcl-2, they vary in cytotoxicity, Bim_S being the most potent.

EXAMPLE 6

Bim binds to and antagonises Bcl-x_L and Bcl-w but not viral Bcl-2 homologs

To determine whether Bim interacts with other members of the Bcl-2 family, we performed immunoprecipitation on lysates from 293T cells transiently co-transfected with the relevant vectors. No interaction with the pro-apoptotic Bax protein was observed, under conditions in which Bax:Bcl-x_L association was readily detectable (data not shown). Association of Bim with Bcl-x_L or each of three point mutants was assessed in ^{35}S-labelled 293T cells (Figure 7A). Bim bound to wild-type Bcl-x_L but not to a mutant (mt 7) that lacks pro-survival activity, nor to two mutants (mt 1 and mt 15) which retain significant anti-apoptotic activity but cannot bind to Bax (Cheng *et al.*, 1996).

Bim_L also bound strongly to the other cellular pro-survival regulator tested, Bcl-w (Gibson *et al.*, 1996) (Figure 7B). In marked contrast, Bim_L did not bind to either of two virally encoded Bcl-2 homologs, the adenovirus E1B19K protein (White *et al.*, 1992) (Figure 7B) and the Epstein-Barr virus BHRF-1 protein (Henderson *et al.*, 1993) (data not shown), even though both viral proteins bound to EE-Bax. Thus, not all mediators of cell survival associate with Bim.

Functional tests mirrored the binding properties of the various Bcl-2 homologs. When transiently co-expressed with Bim in 293T cells, Bcl-x_L and Bcl-w countered Bim toxicity as effectively as Bcl-2 (Figures 7C and 7D). In contrast, little inhibition was observed with comparable levels of the mutant Bcl-x_L proteins (Figure 7C) or the adenovirus E1B19K protein (Figure 7D). These data suggest that Bcl-2-like inhibitors of apoptosis must bind to Bim to inhibit its action.

EXAMPLE 7

The BH3 region is essential for interaction of Bim with Bcl-2 and for most of its ability to promote apoptosis

Since the BH3 region of several death-promoting proteins is essential for their activity (see Introduction), we tested a *bim_L* mutant lacking the BH3 region. In transfected cells the mutant protein (ÆBH3) was readily detected by immunofluorescence and Western blotting (Figure 8A and data not shown), establishing that BH3 is not essential for stability of the polypeptide. Unlike wt Bim, however, the ÆBH3 mutant did not bind to Bcl-2 *in vivo* (Figure 8B).

In some biological assays, the ÆBH3 mutant of Bim appeared inert. In contrast to wt Bim, it was easy to establish lines expressing Bim_L ÆBH3 from FDC-P1 (Figure 8A) or L929 cells (Table 1 and data not shown). Moreover, Bim_L ΔBH3 did not impair the viability of the FDC-P1 cells in either the presence or absence of Bcl-2 (Figure 8C). Finally, 293T cells transiently transfected with Bim_L ΔBH3 exhibited high viability (not

shown). These results indicate that the BH3 region is critical for Bim to promote apoptosis and suggest that Bcl-2 blocks this activity of Bim by binding to that domain. Importantly, however, Bim_L ÆBH3 was not completely inactive. In the L929 clonogenicity assay, it still markedly suppressed colony formation (Table 1). Thus, regions of Bim other than BH3 may promote apoptosis or interfere with clonogenicity in another way, such as by blocking cell growth.

EXAMPLE 8

Expression library screening and isolation of mouse and human bim cDNAs

Polyadenylated RNA prepared from $p53^{-/-}$ KO52DA20 T lymphoma (Strasser *et al.*, 1994) cells subjected to γ-irradiation (10 Gy) was reverse-transcribed, using a combination of oligo dT and random oligonucleotide primers, and ligated to EcoRI adaptors, using standard procedures. The cDNA was then ligated with Eco RI + Xho I-digested λ ZapExpress (Stratagene) arms and packaged *in vitro* according to the supplier's instructions. The resulting expression library was screened using radiolabelled Bcl-2 lacking the hydrophobic membrane localisation region. To prepare this probe, cDNA encoding amino acids 1 to 210 of human Bcl-2 was subcloned into the vector pARΔR1 (Blanar and Rutter, 1992), and recombinant protein (FLAG-HMK-Bcl-2ÆC30) produced in IPTG-induced *E. coli* BL21pLysS (DE3) cells (Novagen) was purified on anti-FLAG M2 affinity gel (IBI Kodak) and then kinased *in vitro* using bovine heart muscle kinase (Sigma) and [γ-^{32}P]ATP (Amersham) (Blanar and Rutter, 1992). ~10^6 plaques were screened with ~10^7 cpm of the radiolabelled probe using the protocol of Blanar and Rutter (Blanar and Rutter, 1992). To reduce non-specific background, the filters were pre-incubated with lysates from induced parental BL21pLysS (DE3) cells and excess unlabelled ATP. Plaques that were positive on duplicate lifts were picked for two rounds of further screening. Positive clones were excised *in vivo* by coinfection with filamentous ExAssist (Stratagene) helper phage and sequenced by automated sequencing (ABI Perkin Elmer). The human *bim* cDNA clone was isolated by screening human embryo and liver λ cDNA libraries

(Stratagene) with an ~800bp mouse *bim* cDNA probe, using standard techniques. The cDNAs were fully sequenced, analysed using Wisconsin GCG or DNASTAR software and compared with sequences in the Genbank (including dBEST) and EMBL databases using the BLAST algorithm (Altschul *et al.*, 1990).

EXAMPLE 9

Expression constructs and site-directed mutagenesis

cDNAs were cloned into the expression vectors pEF PGKpuro (Huang *et al.*, 1997) or pEF PGKhygro (Huang *et al.*, 1997), or derivatives thereof incorporating N-terminal FLAG (DYKDDDK) SEQ ID NO: 11 (Hopp *et al.*, 1988) or EE (EYPME) SEQ ID NO: 12 (Grussenmeyer *et al.*, 1985) epitope tags. The *bim*ÆBH3 mutation was generated by deleting the DNA encoding amino acids 94 to 100 (LRRIGDE) SEQ ID NO: 13and replacing this with DNA corresponding to a Hind III site (encoding GS). Mutations in *bcl-2* (ΔBH4, G145E, W188A) (O'Reilly *et al.*, 1996; Huang *et al.*, 1997) were generated by polymerase chain reaction via splice overlap extension (Horton *et al.*, 1993) using the proof-reading *Pfu* DNA polymerase (Stratagene) (oligonucleotides used are detailed in SEQ ID NO: 14-26). The sequences of derived clones were verified by automated sequencing prior to function analysis.

EXAMPLE 10

Cell culture and transfection

Cell lines used were: mouse IL-3-dependent promyelocytic line FDC-P1; mouse T hybridoma B6.2.16BW2; mouse B lymphoma lines CH1 and WEHI 231; mouse pre-B lymphoma line WEHI 415 (derived from a tumour which arose in an Eμ-*myc* transgenic mouse); human B lymphoblastoid line SKW6; human T lymphoma line Jurkat; mouse T lymphoma lines WEHI 703, WEHI 707 (both derived from tumours which arose in Eμ-NRas transgenic mice) and WEHI 7.1; rat fibroblastoid line Rat-1; mouse fibroblastoid line NIH 3T3; mouse fibroblastoid line L929 subline LM(-TK); human

embryonal kidney cell line 293 (ATCC CRL-1573) and SV40-transformed 293 cells, 293T (see Lithgow *et al.*, 1994; Strasser *et al.*, 1994; Strasser *et al.*, 1995; Huang *et al.*, 1997). The procedures for culture and stable transfection are described elsewhere (Huang *et al.*, 1997). Drug-resistant transfectants were cloned using the cell deposition unit of a FACStarPlus (Becton Dickinson) and clones expressing high levels of the protein of interest were identified by immunofluorescence staining of fixed and permeabilised cells followed by flow cytometric analysis.

EXAMPLE 11

Cell death assays

Cytokine deprivation and exposure to ionising radiation were the principal cell death assays used to assess the sensitivity of FDC-P1 cells stably transfected with the various expression vectors. Cells were cultured in medium lacking cytokine or (in complete medium) after exposure to 10 Gy γ-radiation (provided by a ^{60}Co source at a rate of 3 Gy/min) and their viability determined over several days by vital dye (0.4% eosin) exclusion, as assessed by visual inspection in a hemocytometer, or by flow cytometric analysis of cells that excluded propidium iodide (5μg/ml; Sigma) (Nicoletti *et al.*, 1991).

Cell death assays in 293T cells were performed after transient transfection of $\sim 5 \times 10^5$ cells using 6 μl of Lipofectamine® (Gibco BRL) and a total of 1 μg DNA in 2 ml of medium in 6 cm dishes; for co-transfections, *bim* plasmid (0.1, 0.2, 0.5 μg) was co-transfected with 0.5μg of the other recombinant (eg *bcl-2*) plasmid and (0.4, 0.2, 0 μg) of empty vector. Forty-eight hours after transfection, the cells were harvested, fixed for 5 min in 80% methanol, permeabilised with 0.3% saponin (which was included in all the subsequent steps), and stained with 1 μg/ml anti-EE monoclonal antibody (BabCO), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1 μg/ml; Southern Biotechnology) as the secondary agent and by 69 μM propidium iodide in 38 mM sodium citrate pH 7.4 (Crissman *et al.*, 1990). Analysis

was performed on a FACScan II (Becton Dickinson), the proportion of dead cells being taken to be the proportion of EE-positive cells with less than 2C DNA content (Nicoletti *et al.*, 1991).

L929 fibroblast colony assays were performed in triplicate by scoring the numbers of colonies in 10 cm dishes grown for 14-18 days with appropriate antibiotic selection. These cells had previously been split (1:3) from $\sim 10^6$ cells which had been transfected in 6 cm dishes 2 days earlier with 1 μg total DNA and 12 μl of Lipofectamine®.

EXAMPLE 12

Immunofluorescence, immunoprecipitation and immunoblotting

Immunofluorescence staining of cytoplasmic proteins with the monoclonal antibodies Bcl-2-100 (mouse anti-human Bcl-2; [Pezzella *et al.*, 1990]) or mouse anti-EE (BabCO) followed by FITC-conjugated goat anti-mouse IgG (Southern Biotechnology) was performed as previously described (Huang *et al.*, 1997). Cells were analysed in the FACScan II (Becton Dickinson) after exclusion of dead cells on the basis of their forward and side scatter characteristics.

To investigate the subcellular localisation of EE-tagged Bim_L, transfected L929 fibroblasts grown in chamber slides (Erie Scientific Company, New Hampshire) were fixed in 4% paraformaldehyde for 10 min at room temperature and the slides were then allowed to dry and stored at -20°C. Prior to analysis by confocal microscopy, the cells were rehydrated by dipping the slides in water and then permeabilised for 15 minutes at room temperature in 0.5% Triton-X 100 in PBS. EE-Bim_L was detected by incubating for the cells with anti-EE monoclonal antibody for 30 minutes, washing several times in PBS containing 2% foetal calf serum and 0.05% Tween-20, and then incubating for 30 minutes with goat anti-mouse IgG conjugated to lissamine-rhodamine (Jackson Immunoresearch), all steps being performed at room temperature. Human Bcl-2 was detected similarly, using hamster anti-human Bcl-2 (6C8) (Veis *et al.*, 1993) followed

by FITC-conjugated mouse anti-hamster IgG. Untransfected cells served as negative controls. Samples were analysed using a Leica confocal laser scanning microscope (Leica Lasertechnik).

To test for protein-protein interactions *in vivo*, immunoblotting was performed on stably transfected FDC-P1 cells or transiently transfected 293T cells as described previously (Huang *et al.*, 1997). Briefly, lysates prepared from 10^5-10^6 cells were incubated with ~5 μg antibody (anti-human Bcl-2, anti-FLAG M2 (IBI Kodak), or anti-EE monoclonal antibody), followed by protein G Sepharose (Pharmacia), and then pelleted, washed, fractionated by SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. The filters were incubated with mouse anti-human Bcl-2, anti-FLAG or anti-EE antibodies followed by affinity-purified rabbit anti-mouse IgG; bound antibodies were detected with ^{125}I-labelled staphylococcal protein A. In some experiments, the cells were metabolically labelled with 100-200 μCi/ml of ^{35}S-methionine (NEG-072 from NEN) and equivalent TCA-precipitable counts ($5x10^6$ cpm) were used for each immunoprecipitation.

Dated this 17th day of September 1997.

The Walter and Eliza Hall Institute of Medical Research
by its Patent Attorneys
DAVIES COLLISON CAVE

Table 1 Bim inhibition of L929 colony growth is abrogated by Bcl-2

Cell line	Construct	Cloning efficiency	Number of clones expressing Bim
L929			
	control	1.0	0/6
	bim_L	0.21 ± 0.04	1/6
	bim_{EL}	0.19 ± 0.05	1/6
	bim_S	0.11 ± 0.03	0/6
	bim_L $\Delta BH3$	0.69 ± 0.07	6/6
L929 *bcl-2*			
	control	1.0	0/6
	bim_L	0.64 ± 0.07	4/6

Parental L929 fibroblasts and a cloned derivative that stably expresses human Bcl-2 (L929 *bcl-2*) were co-transfected with a plasmid encoding a puromycin resistance gene with or without various forms of Bim. Forty eight hours later, puromycin selection was added and the number of colonies were scored after 14 to 18 days. The data shown are means ± SD of at least 4 independent experiments.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH

(ii) TITLE OF INVENTION: NOVEL THERAPEUTIC MOLECULES

(iii) NUMBER OF SEQUENCES: 26

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: AUSTRALIAN PROVISIONAL APPLICATION
(B) FILING DATE:
(C) CLASSIFICATION:

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(C) TELEX: AA 31787

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 333 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..333

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GGA CAA TTG CAG CCT GCT GAG AGG CCT CCC CAG CTC AGG CCT GGG GCC
96
Gly Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
             20                  25                  30

CCT ACC TCC CTA CAG ACA GAA CCG CAA GCT TCC ATA CGA CAG TCT CAG
144
Pro Thr Ser Leu Gln Thr Glu Pro Gln Ala Ser Ile Arg Gln Ser Gln
         35                  40                  45

GAG GAA CCT GAA GAT CTG CGC CCG GAG ATA CGG ATT GCA CAG GAG CTG
192
Glu Glu Pro Glu Asp Leu Arg Pro Glu Ile Arg Ile Ala Gln Glu Leu
     50                  55                  60

CGG CGG ATC GGA GAC GAG TTC AAC GAA ACT TAC ACA AGG AGG GTG TTT
240
Arg Arg Ile Gly Asp Glu Phe Asn Glu Thr Tyr Thr Arg Arg Val Phe
 65                  70                  75                  80

GCA AAT GAT TAC CGC GAG GCT GAA GAC CAC CCT CAA ATG GTT ATC TTA
288
Ala Asn Asp Tyr Arg Glu Ala Glu Asp His Pro Gln Met Val Ile Leu
                 85                  90                  95

CAA CTG TTA CGC TTT ATC TTC CGT CTG GTA TGG AGA AGG CAT TG
333
Gln Leu Leu Arg Phe Ile Phe Arg Leu Val Trp Arg Arg His
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 1               5                  10                  15

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            20                  25                  30

Pro Thr Ser Leu Gln Thr Glu Pro Gln Ala Ser Ile Arg Gln Ser Gln
        35                  40                  45

Glu Glu Pro Glu Asp Leu Arg Pro Glu Ile Arg Ile Ala Gln Glu Leu
    50                  55                  60

Arg Arg Ile Gly Asp Glu Phe Asn Glu Thr Tyr Thr Arg Arg Val Phe
65                  70                  75                  80

Ala Asn Asp Tyr Arg Glu Ala Glu Asp His Pro Gln Met Val Ile Leu
                85                  90                  95

Gln Leu Leu Arg Phe Ile Phe Arg Leu Val Trp Arg Arg His
            100                 105                 110
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 423 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..423

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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48
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 1               5                  10                  15

GGA CAA TTG CAG CCT GCT GAG AGG CCT CCC CAG CTC AGG CCT GGG GCC
96
```

```
Gly Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
            20                  25                  30

CCT ACC TCC CTA CAG ACA GAA CCG CAA GAC AGG AGC CCG GCA CCC ATG
144
Pro Thr Ser Leu Gln Thr Glu Pro Gln Asp Arg Ser Pro Ala Pro Met
        35                  40                  45

AGT TGT GAC AAG TCA ACA CAA ACC CCA AGT CCT CCT TGC CAG GCC TTC
192
Ser Cys Asp Lys Ser Thr Gln Thr Pro Ser Pro Pro Cys Gln Ala Phe
    50                  55                  60

AAC CAC TAT CTC AGT GCA ATG GCT TCC ATA CGA CAG TCT CAG GAG GAA
240
Asn His Tyr Leu Ser Ala Met Ala Ser Ile Arg Gln Ser Gln Glu Glu
65                  70                  75                  80

CCT GAA GAT CTG CGC CCG GAG ATA CGG ATT GCA CAG GAG CTG CGG CGG
288
Pro Glu Asp Leu Arg Pro Glu Ile Arg Ile Ala Gln Glu Leu Arg Arg
                85                  90                  95

ATC GGA GAC GAG TTC AAC GAA ACT TAC ACA AGG AGG GTG TTT GCA AAT
336
Ile Gly Asp Glu Phe Asn Glu Thr Tyr Thr Arg Arg Val Phe Ala Asn
            100                 105                 110

GAT TAC CGC GAG GCT GAA GAC CAC CCT CAA ATG GTT ATC TTA CAA CTG
384
Asp Tyr Arg Glu Ala Glu Asp His Pro Gln Met Val Ile Leu Gln Leu
        115                 120                 125

TTA CGC TTT ATC TTC CGT CTG GTA TGG AGA AGG CAT TG
423
Leu Arg Phe Ile Phe Arg Leu Val Trp Arg Arg His
    130                 135                 140
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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  1               5                  10                  15

Gly Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
            20                  25                  30
```

```
Pro Thr Ser Leu Gln Thr Glu Pro Gln Asp Arg Ser Pro Ala Pro Met
         35                  40                  45

Ser Cys Asp Lys Ser Thr Gln Thr Pro Ser Pro Pro Cys Gln Ala Phe
     50                  55                  60

Asn His Tyr Leu Ser Ala Met Ala Ser Ile Arg Gln Ser Gln Glu Glu
 65                  70                  75                  80

Pro Glu Asp Leu Arg Pro Glu Ile Arg Ile Ala Gln Glu Leu Arg Arg
                85                  90                  95

Ile Gly Asp Glu Phe Asn Glu Thr Tyr Thr Arg Arg Val Phe Ala Asn
            100                 105                 110

Asp Tyr Arg Glu Ala Glu Asp His Pro Gln Met Val Ile Leu Gln Leu
        115                 120                 125

Leu Arg Phe Ile Phe Arg Leu Val Trp Arg Arg His
    130                 135                 140
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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 591 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..591

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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48
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  1               5                  10                  15

GGA CAA TTG CAG CCT GCT GAG AGG CCT CCC CAG CTC AGG CCT GGG GCC
96
Gly Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
            20                  25                  30

CCT ACC TCC CTA CAG ACA GAA CCG CAA GGT AAT CCC GAC GGC GAA GGG
144
Pro Thr Ser Leu Gln Thr Glu Pro Gln Gly Asn Pro Asp Gly Glu Gly
         35                  40                  45

GAC CGC TGC CCC CAC GGC AGC CCT CAG GGC CCG CTG GCC CCA CCG GCC
192
```

```
Asp Arg Cys Pro His Gly Ser Pro Gln Gly Pro Leu Ala Pro Pro Ala
     50                  55                  60

AGC CCT GGC CCT TTT GCT ACC AGA TCC CCA CTT TTC ATC TTT GTG AGA
240
Ser Pro Gly Pro Phe Ala Thr Arg Ser Pro Leu Phe Ile Phe Val Arg
 65                  70                  75                  80

AGA TCT TCT CTG CTG TCC CGG TCC TCC AGT GGG TAT TTC TCT TTT GAC
288
Arg Ser Ser Leu Leu Ser Arg Ser Ser Ser Gly Tyr Phe Ser Phe Asp
                85                  90                  95

ACA GAC AGG AGC CCG GCA CCC ATG AGT TGT GAC AAG TCA ACA CAA ACC
336
Thr Asp Arg Ser Pro Ala Pro Met Ser Cys Asp Lys Ser Thr Gln Thr
            100                 105                 110

CCA AGT CCT CCT TGC CAG GCC TTC AAC CAC TAT CTC AGT GCA ATG GCT
384
Pro Ser Pro Pro Cys Gln Ala Phe Asn His Tyr Leu Ser Ala Met Ala
        115                 120                 125

TCC ATA CGA CAG TCT CAG GAG GAA CCT GAA GAT CTG CGC CCG GAG ATA
432
Ser Ile Arg Gln Ser Gln Glu Glu Pro Glu Asp Leu Arg Pro Glu Ile
    130                 135                 140

CGG ATT GCA CAG GAG CTG CGG CGG ATC GGA GAC GAG TTC AAC GAA ACT
480
Arg Ile Ala Gln Glu Leu Arg Arg Ile Gly Asp Glu Phe Asn Glu Thr
145                 150                 155                 160

TAC ACA AGG AGG GTG TTT GCA AAT GAT TAC CGC GAG GCT GAA GAC CAC
528
Tyr Thr Arg Arg Val Phe Ala Asn Asp Tyr Arg Glu Ala Glu Asp His
                165                 170                 175

CCT CAA ATG GTT ATC TTA CAA CTG TTA CGC TTT ATC TTC CGT CTG GTA
576
Pro Gln Met Val Ile Leu Gln Leu Leu Arg Phe Ile Phe Arg Leu Val
            180                 185                 190

TGG AGA AGG CAT TG
591
Trp Arg Arg His
        195
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 196 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Ala Lys Gln Pro Ser Asp Val Ser Ser Glu Cys Asp Arg Glu Gly
 1               5                  10                  15

Gly Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
            20                  25                  30

Pro Thr Ser Leu Gln Thr Glu Pro Gln Gly Asn Pro Asp Gly Glu Gly
        35                  40                  45

Asp Arg Cys Pro His Gly Ser Pro Gln Gly Pro Leu Ala Pro Pro Ala
    50                  55                  60

Ser Pro Gly Pro Phe Ala Thr Arg Ser Pro Leu Phe Ile Phe Val Arg
65                  70                  75                  80

Arg Ser Ser Leu Leu Ser Arg Ser Ser Ser Gly Tyr Phe Ser Phe Asp
                85                  90                  95

Thr Asp Arg Ser Pro Ala Pro Met Ser Cys Asp Lys Ser Thr Gln Thr
            100                 105                 110

Pro Ser Pro Pro Cys Gln Ala Phe Asn His Tyr Leu Ser Ala Met Ala
        115                 120                 125

Ser Ile Arg Gln Ser Gln Glu Glu Pro Glu Asp Leu Arg Pro Glu Ile
    130                 135                 140

Arg Ile Ala Gln Glu Leu Arg Arg Ile Gly Asp Glu Phe Asn Glu Thr
145                 150                 155                 160

Tyr Thr Arg Arg Val Phe Ala Asn Asp Tyr Arg Glu Ala Glu Asp His
                165                 170                 175

Pro Gln Met Val Ile Leu Gln Leu Leu Arg Phe Ile Phe Arg Leu Val
            180                 185                 190

Trp Arg Arg His
        195
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 417 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..417

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATG GCA AAG CAA CCT TCT GAT GTA AGT TCT GAG TGT GAC CGA GAA GGT
48
Met Ala Lys Gln Pro Ser Asp Val Ser Ser Glu Cys Asp Arg Glu Gly
  1               5                  10                  15

AGA CAA TTG CAG CCT GCG GAG AGG CCT CCC CAG CTC AGA CCT GGG GCC
96
Arg Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
            20                  25                  30

CCT ACC TCC CTA CAG ACA GAG CCA CAA GAC AGG AGC CCA GCA CCC ATG
144
Pro Thr Ser Leu Gln Thr Glu Pro Gln Asp Arg Ser Pro Ala Pro Met
        35                  40                  45

AGT TGT GAC AAA TCA ACA CAA ACC CCA AGT CCT CCT TGC CAG GCC TTC
192
Ser Cys Asp Lys Ser Thr Gln Thr Pro Ser Pro Pro Cys Gln Ala Phe
    50                  55                  60

AAC CAC TAT CTC AGT GCA ATG GCT TCC ATG AGG CAG GCT GAA CCT GCA
240
Asn His Tyr Leu Ser Ala Met Ala Ser Met Arg Gln Ala Glu Pro Ala
65                  70                  75                  80

GAT ATG CGC CCA GAG ATA TGG ATC GCC CAA GAG TTG CGG CGT ATC GGA
288
Asp Met Arg Pro Glu Ile Trp Ile Ala Gln Glu Leu Arg Arg Ile Gly
                85                  90                  95

GAC GAG TTT AAC GCT TAC TAT GCA AGG AGG GTA TTT TTG AAT AAT TAC
336
Asp Glu Phe Asn Ala Tyr Tyr Ala Arg Arg Val Phe Leu Asn Asn Tyr
            100                 105                 110

CAA GCA GCC GAA GAC CAC CCA CGA ATG GTT ATC TTA CGA CTG TTA CGT
384
Gln Ala Ala Glu Asp His Pro Arg Met Val Ile Leu Arg Leu Leu Arg
        115                 120                 125
```

```
TAC ATT GTC CGC CTG GTG TGG AGA ATG CAT TG
417
Tyr Ile Val Arg Leu Val Trp Arg Met His
    130                 135
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 138 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Ala Lys Gln Pro Ser Asp Val Ser Ser Glu Cys Asp Arg Glu Gly
 1               5                  10                  15

Arg Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
            20                  25                  30

Pro Thr Ser Leu Gln Thr Glu Pro Gln Asp Arg Ser Pro Ala Pro Met
        35                  40                  45

Ser Cys Asp Lys Ser Thr Gln Thr Pro Ser Pro Pro Cys Gln Ala Phe
    50                  55                  60

Asn His Tyr Leu Ser Ala Met Ala Ser Met Arg Gln Ala Glu Pro Ala
65                  70                  75                  80

Asp Met Arg Pro Glu Ile Trp Ile Ala Gln Glu Leu Arg Arg Ile Gly
                85                  90                  95

Asp Glu Phe Asn Ala Tyr Tyr Ala Arg Arg Val Phe Leu Asn Asn Tyr
            100                 105                 110

Gln Ala Ala Glu Asp His Pro Arg Met Val Ile Leu Arg Leu Leu Arg
        115                 120                 125

Tyr Ile Val Arg Leu Val Trp Arg Met His
    130                 135
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 597 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..597

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
ATG GCA AAG CAA CCT TCT GAT GTA AGT TCT GAG TGT GAC CGA GAA GGT
48
Met Ala Lys Gln Pro Ser Asp Val Ser Ser Glu Cys Asp Arg Glu Gly
  1               5                  10                  15

AGA CAA TTG CAG CCT GCG GAG AGG CCT CCC CAG CTC AGA CCT GGG GCC
96
Arg Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
            20                  25                  30

CCT ACC TCC CTA CAG ACA GAG CCA CAA GGT AAT CCT GAA GGC AAT CAC
144
Pro Thr Ser Leu Gln Thr Glu Pro Gln Gly Asn Pro Glu Gly Asn His
        35                  40                  45

GGA GGT GAA GGG GAC AGC TGC CCC CAC GGC AGC CCT CAG GGC CCG CTG
192
Gly Gly Glu Gly Asp Ser Cys Pro His Gly Ser Pro Gln Gly Pro Leu
    50                  55                  60

GCC CCA CCT GCC AGC CCT GGC CCT TTT GCT ACC AGA TCC CCG CTT TTC
240
Ala Pro Pro Ala Ser Pro Gly Pro Phe Ala Thr Arg Ser Pro Leu Phe
65                  70                  75                  80

ATC TTT ATG AGA AGA TCC TCC CTG CTG TCT CGA TCC TCC AGT GGG TAT
288
Ile Phe Met Arg Arg Ser Ser Leu Leu Ser Arg Ser Ser Ser Gly Tyr
                85                  90                  95

TTC TCT TTT GAC ACA GAC AGG AGC CCA GCA CCC ATG AGT TGT GAC AAA
336
Phe Ser Phe Asp Thr Asp Arg Ser Pro Ala Pro Met Ser Cys Asp Lys
            100                 105                 110

TCA ACA CAA ACC CCA AGT CCT CCT TGC CAG GCC TTC AAC CAC TAT CTC
384
Ser Thr Gln Thr Pro Ser Pro Pro Cys Gln Ala Phe Asn His Tyr Leu
        115                 120                 125
```

```
AGT GCA ATG GCT TCC ATG AGG CAG GCT GAA CCT GCA GAT ATG CGC CCA
432
Ser Ala Met Ala Ser Met Arg Gln Ala Glu Pro Ala Asp Met Arg Pro
    130                 135                 140

GAG ATA TGG ATC GCC CAA GAG TTG CGG CGT ATC GGA GAC GAG TTT AAC
480
Glu Ile Trp Ile Ala Gln Glu Leu Arg Arg Ile Gly Asp Glu Phe Asn
145                 150                 155                 160

GCT TAC TAT GCA AGG AGG GTA TTT TTG AAT AAT TAC CAA GCA GCC GAA
528
Ala Tyr Tyr Ala Arg Arg Val Phe Leu Asn Asn Tyr Gln Ala Ala Glu
                165                 170                 175

GAC CAC CCA CGA ATG GTT ATC TTA CGA CTG TTA CGT TAC ATT GTC CGC
576
Asp His Pro Arg Met Val Ile Leu Arg Leu Leu Arg Tyr Ile Val Arg
            180                 185                 190

CTG GTG TGG AGA ATG CAT TG
597
Leu Val Trp Arg Met His
        195
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 198 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Ala Lys Gln Pro Ser Asp Val Ser Ser Glu Cys Asp Arg Glu Gly
  1               5                  10                  15

Arg Gln Leu Gln Pro Ala Glu Arg Pro Pro Gln Leu Arg Pro Gly Ala
             20                  25                  30

Pro Thr Ser Leu Gln Thr Glu Pro Gln Gly Asn Pro Glu Gly Asn His
         35                  40                  45

Gly Gly Glu Gly Asp Ser Cys Pro His Gly Ser Pro Gln Gly Pro Leu
     50                  55                  60

Ala Pro Pro Ala Ser Pro Gly Pro Phe Ala Thr Arg Ser Pro Leu Phe
 65                  70                  75                  80

Ile Phe Met Arg Arg Ser Ser Leu Leu Ser Arg Ser Ser Ser Gly Tyr
                 85                  90                  95

Phe Ser Phe Asp Thr Asp Arg Ser Pro Ala Pro Met Ser Cys Asp Lys
```

```
           100                 105                 110

Ser Thr Gln Thr Pro Ser Pro Pro Cys Gln Ala Phe Asn His Tyr Leu
        115                 120                 125

Ser Ala Met Ala Ser Met Arg Gln Ala Glu Pro Ala Asp Met Arg Pro
    130                 135                 140

Glu Ile Trp Ile Ala Gln Glu Leu Arg Arg Ile Gly Asp Glu Phe Asn
145                 150                 155                 160

Ala Tyr Tyr Ala Arg Arg Val Phe Leu Asn Asn Tyr Gln Ala Ala Glu
                165                 170                 175

Asp His Pro Arg Met Val Ile Leu Arg Leu Leu Arg Tyr Ile Val Arg
            180                 185                 190

Leu Val Trp Arg Met His
        195
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Asp Tyr Lys Asp Asp Asp Lys
1               5
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Glu Tyr Pro Met Glu
1               5
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Leu Arg Arg Ile Gly Asp Glu
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGGGAGAACA GGGTACATCG ATGCGGG 27

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTGAACTGGG AGCGGATTGT GG 22

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CACCTGCACA CCGCGATCCA GGATAACG 28

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGGATCCACC ATGGCCAAGC AACC 24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTTCTAGATC AGCACATCTC TCTGGGATAG AACCAC 36

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCAAGCTTCCT GTGCAATCCG TATCTCC 27

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGAAGCTTGC AACGAAACTT ACACAAGGTG 30

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCAAGCTTCC GGGCGCAGAT CTTC 24

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CAAAGCTTCC TGTGCAATCC GTATCTCC 28

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGAAGCTTTG AACGAAACTT ACACAAGGTG 30

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CAAAGCTTCC GGGCGCAGAT CTTC 23

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TAAGTTCTGA GTGTGACAGA GAAGGTGG 28

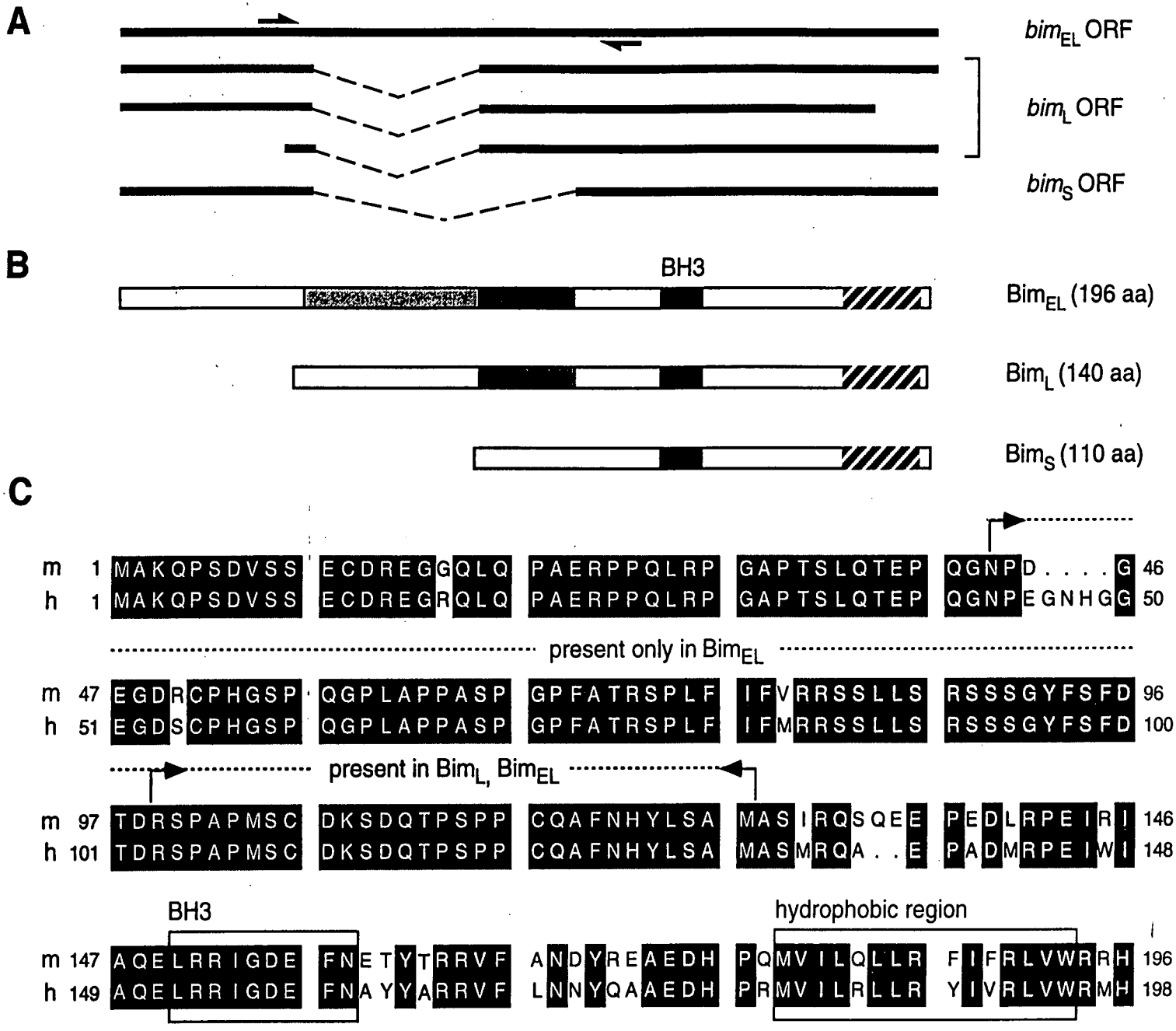
(2) INFORMATION FOR SEQ ID NO:26:

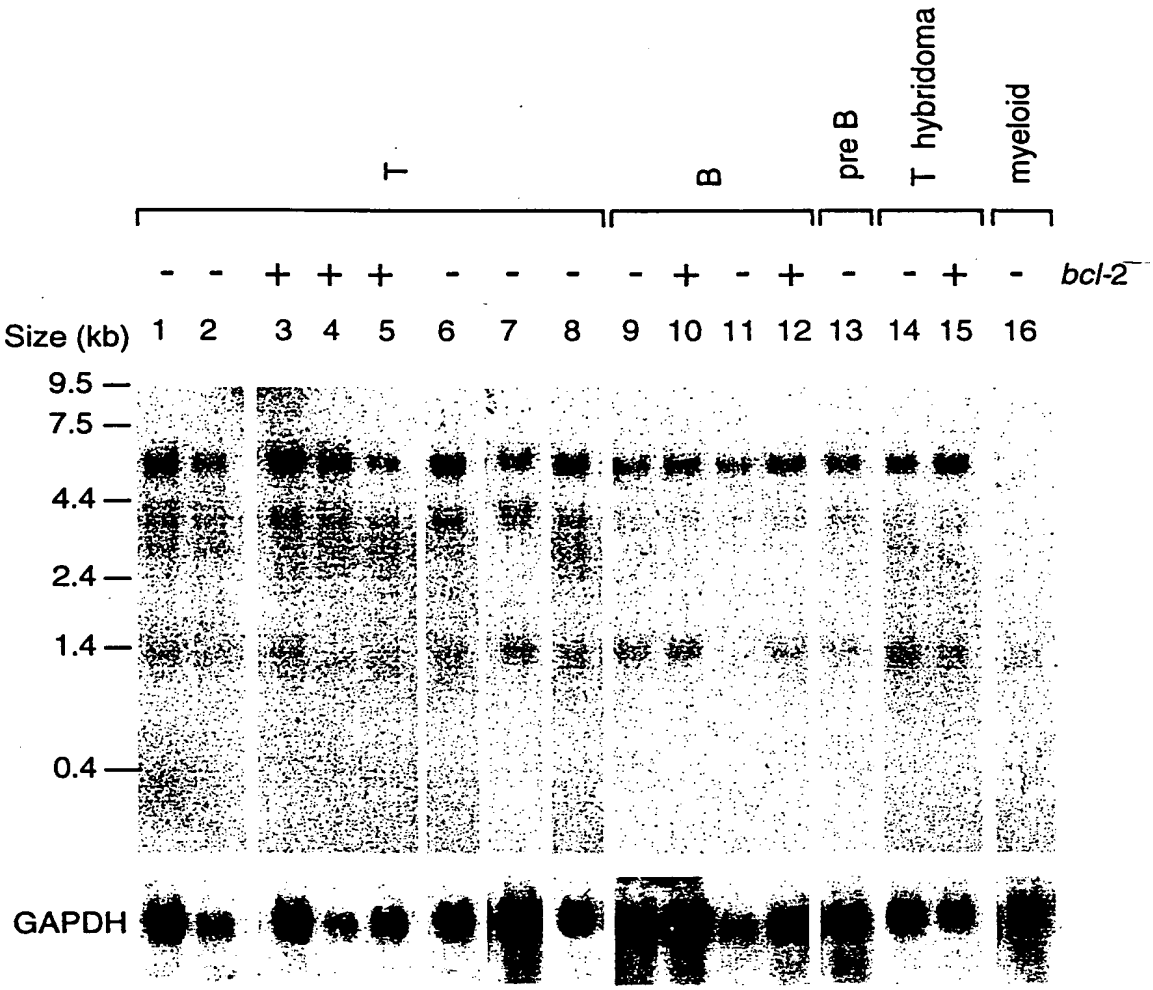
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

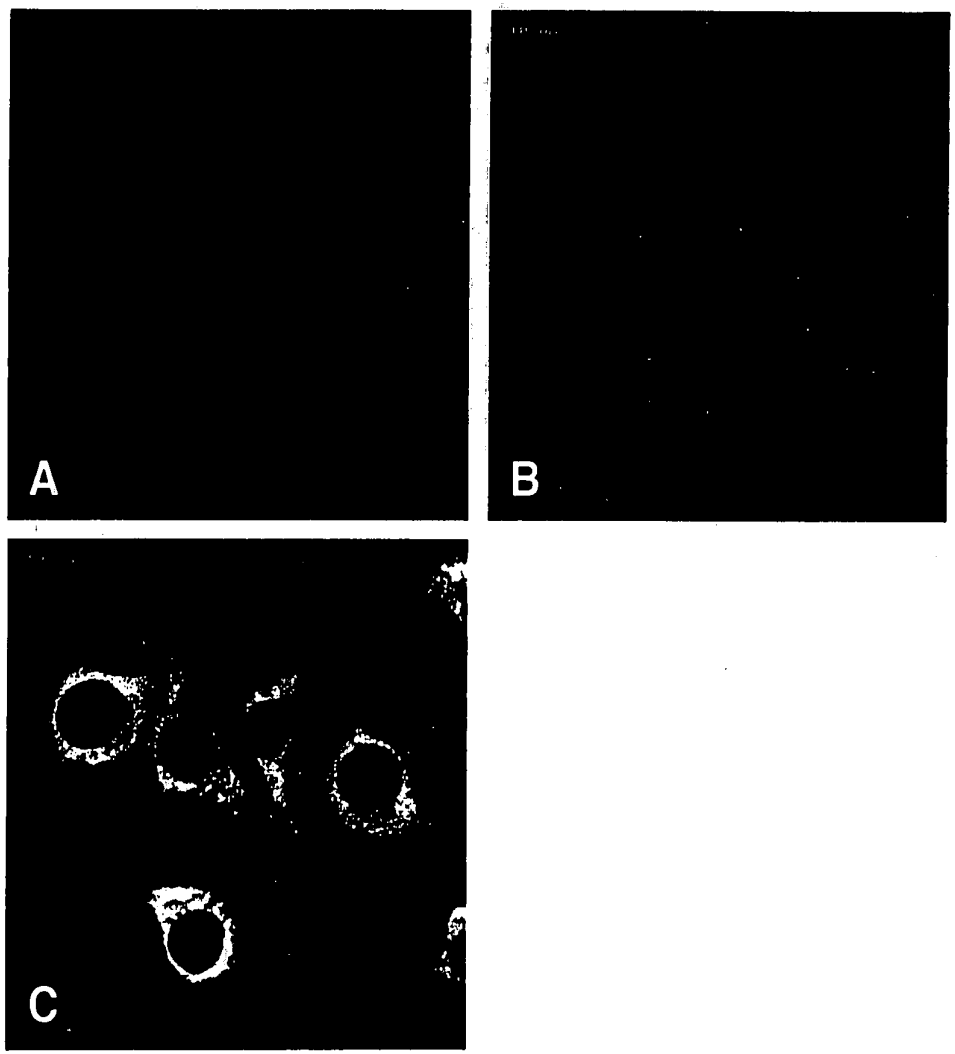
(ii) MOLECULE TYPE: DNA

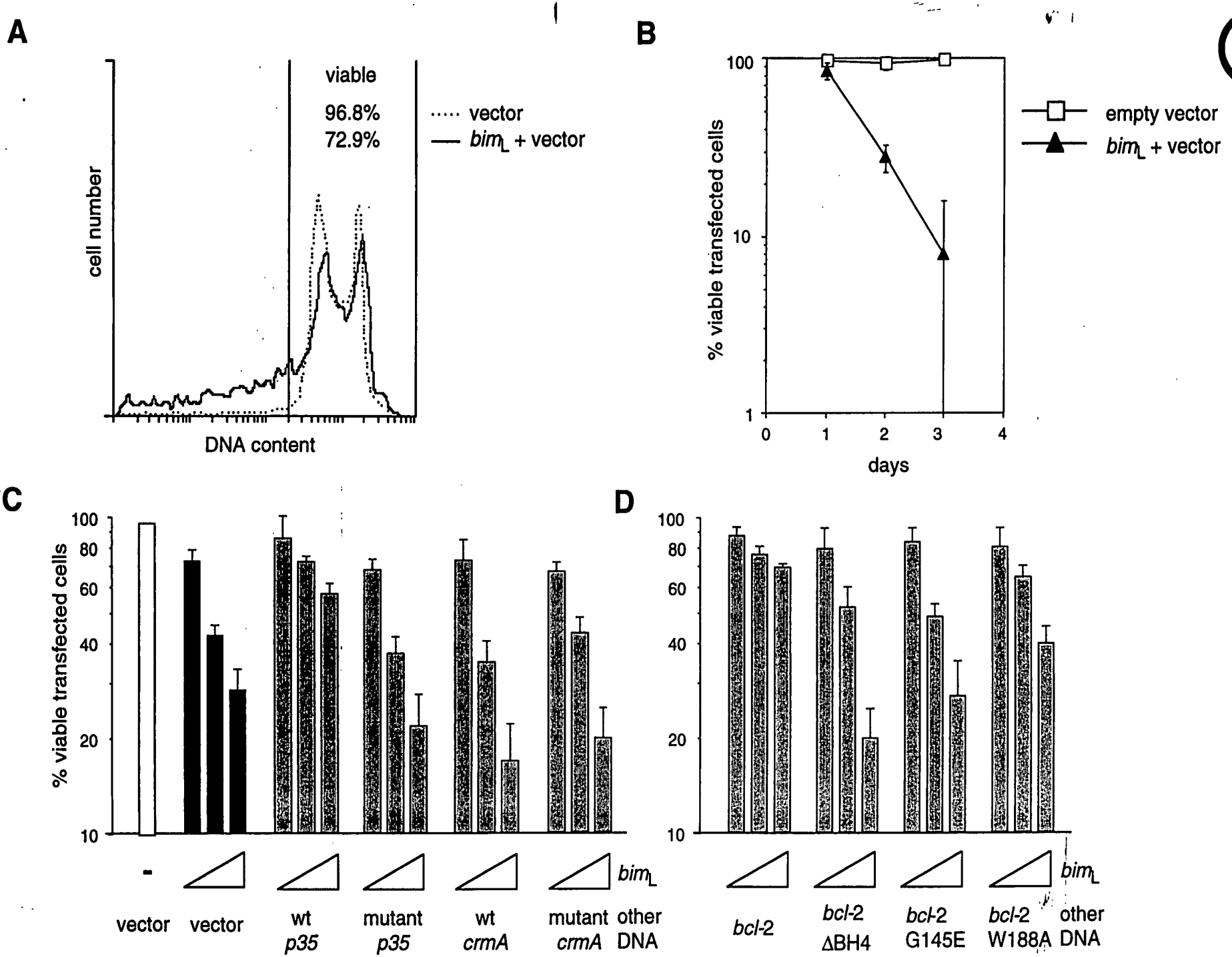
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

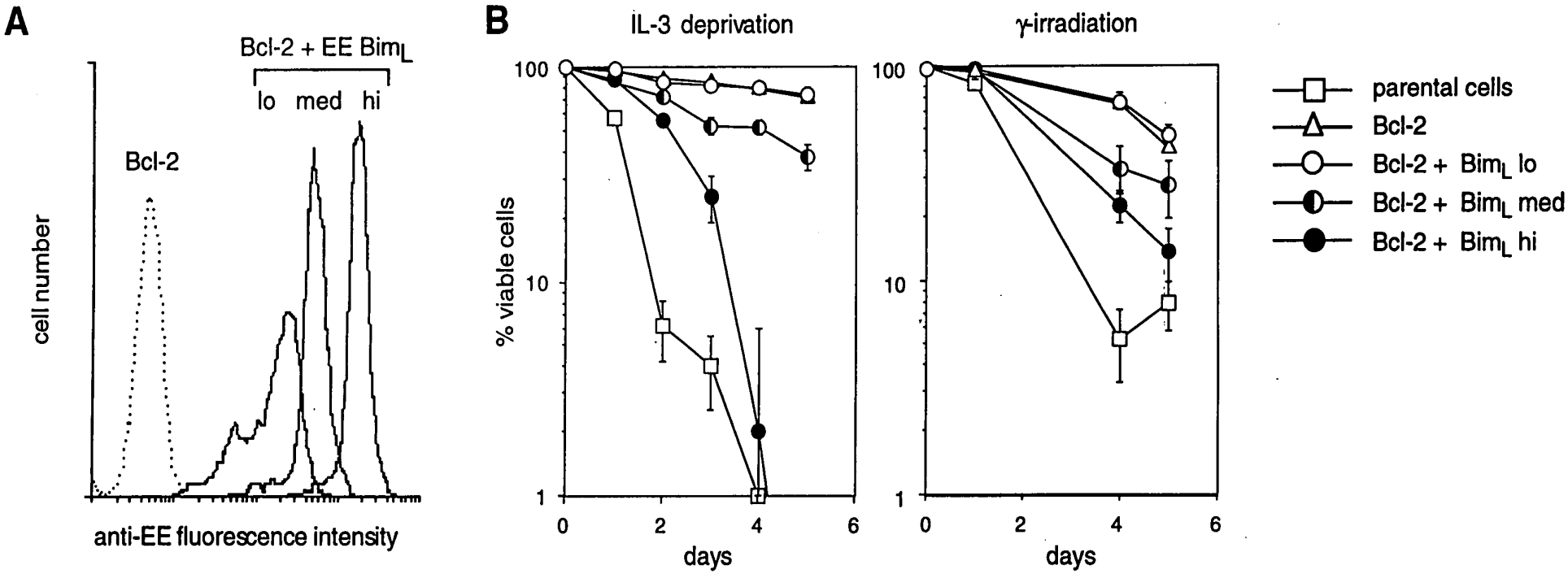
CAGTTGTAAG ATAACCATTT GAGGGTGG 28

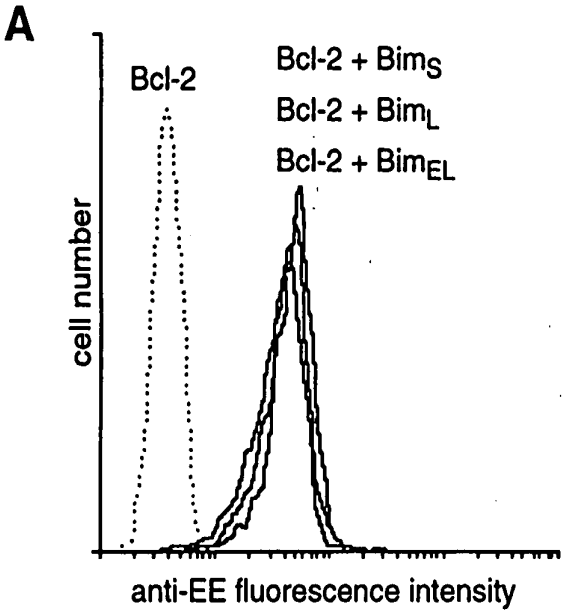

A
bim_EL ORF
bim_L ORF
bim_S ORF
B
BH3
Bim_EL (196 aa)
Bim_L (140 aa)
Bim_S (110 aa)
C
m 1 MAKQPSDVSS ECDREGGQLQ PAERPPQLRP GAPTSLQTEP QGNPD....G 46
h 1 MAKQPSDVSS ECDREGRQLQ PAERPPQLRP GAPTSLQTEP QGNPEGNHGG 50
present only in Bim_EL
m 47 EGDRCPHGSP QGPLAPPASP GPFATRSPLF IFVRRSSLLS RSSSGYFSFD 96
h 51 EGDSCPHGSP QGPLAPPASP GPFATRSPLF IFMRRSSLLS RSSSGYFSFD 100
present in Bim_L, Bim_EL
m 97 TDRSPAPMSC DKSDQTPSPP CQAFNHYLSA MASIRQSQEE PEDLRPEIRI 146
h 101 TDRSPAPMSC DKSDQTPSPP CQAFNHYLSA MASMRQA..E PADMRPEIWI 148
BH3
hydrophobic region
m 147 AQELRRIGDE FNETYTRRVF ANDYREAEDH PQMVILQLLR FIFRLVWRRH 196
h 149 AQELRRIGDE FNAYYARRVF LNNYQAAEDH PRMVILRLLR YIVRLVWRMH 198

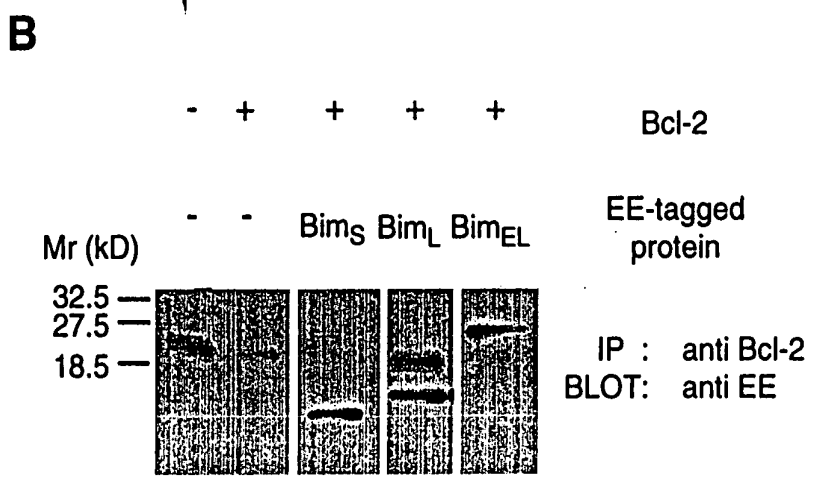

T
B
pre B
T hybridoma
myeloid
- - + + + - - - - + - + - - + - bcl-2
Size (kb) 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
9.5
7.5
4.4
2.4
1.4
0.4
GAPDH

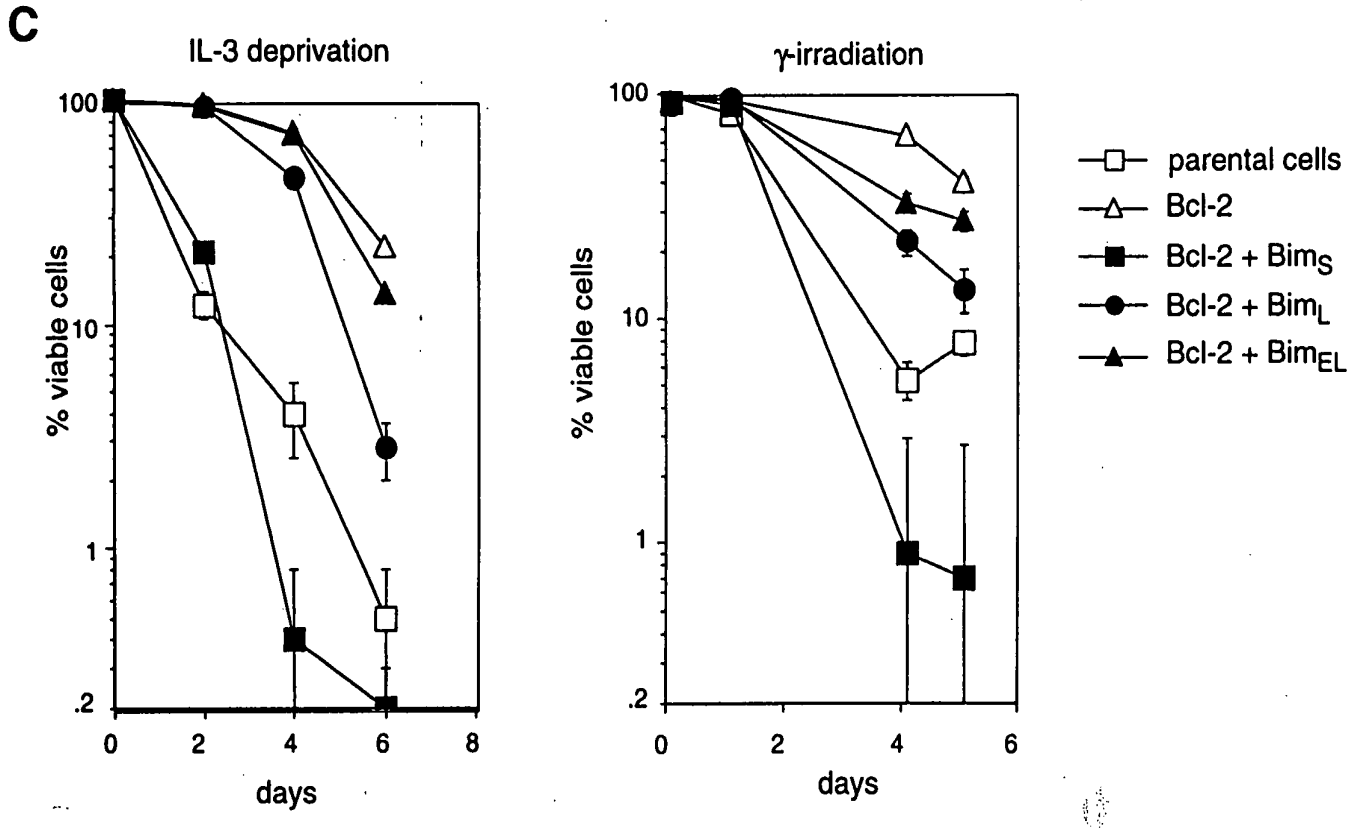

A
B
C

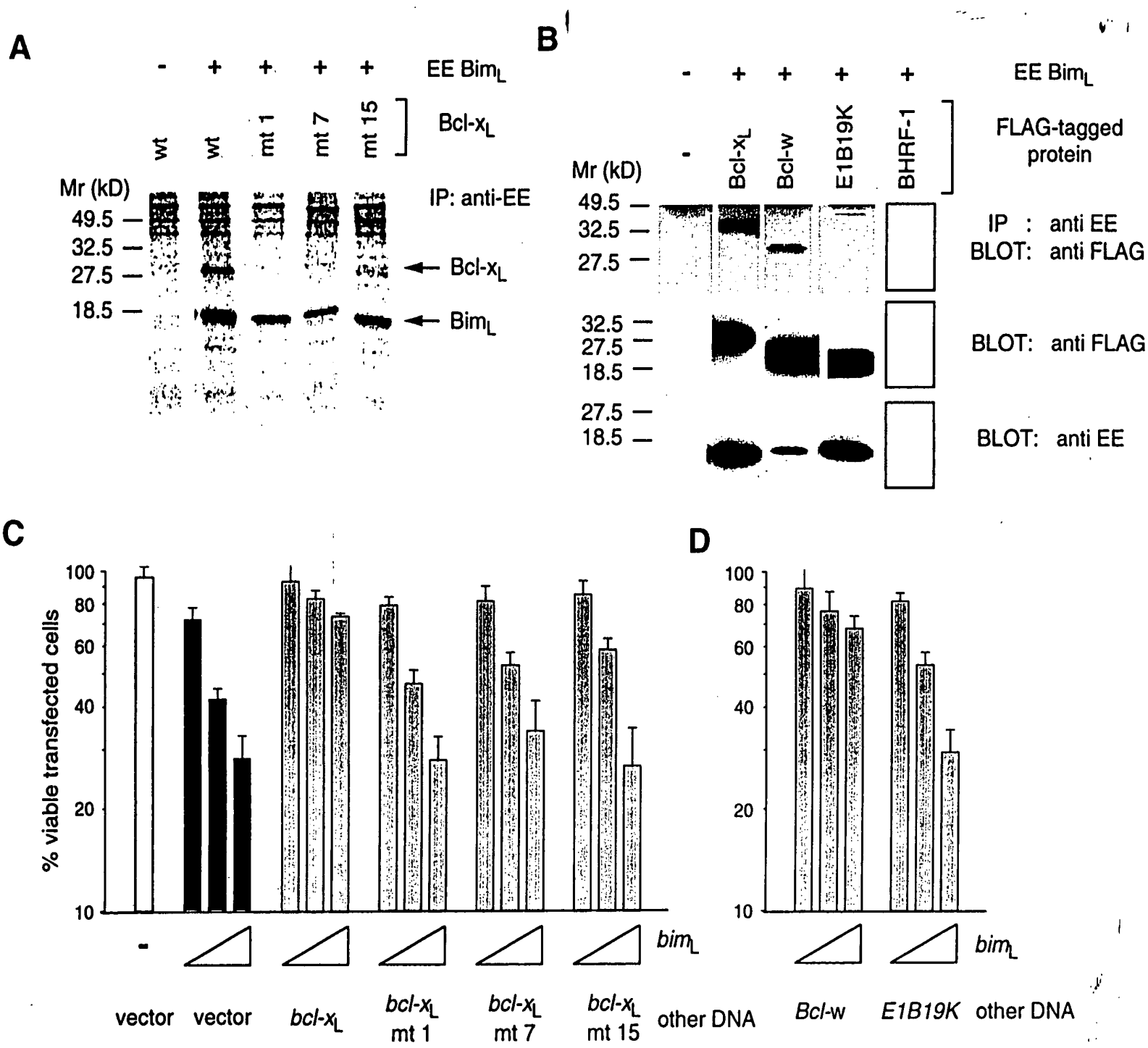

A
viable
96.8%
72.9%
vector
bim$_L$ + vector
cell number
DNA content
B
100
10
1
0
1
2
3
4
days
% viable transfected cells
empty vector
bim$_L$ + vector
C
% viable transfected cells
100
80
60
40
20
10
-
vector
vector
wt p35
mutant p35
wt crmA
mutant crmA
other DNA
bim$_L$
D
bcl-2
bcl-2 ΔBH4
bcl-2 G145E
bcl-2 W188A
other DNA
bim$_L$


A
Bcl-2 + EE Bim$_L$
lo med hi
Bcl-2
cell number
anti-EE fluorescence intensity
B
IL-3 deprivation
γ-irradiation
% viable cells
100
10
1
0
2
4
6
days
parental cells
Bcl-2
Bcl-2 + Bim$_L$ lo
Bcl-2 + Bim$_L$ med
Bcl-2 + Bim$_L$ hi

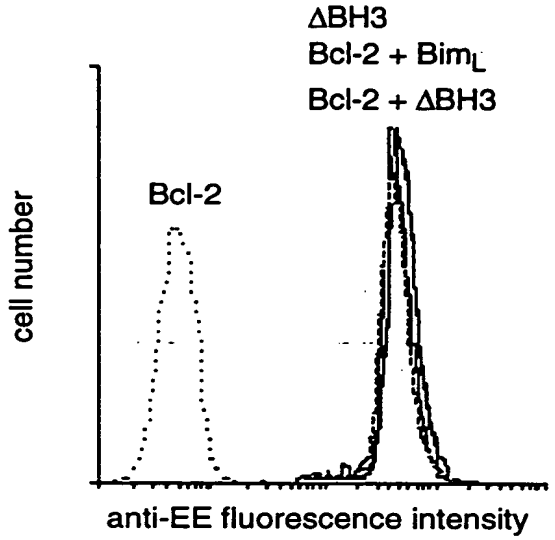

A
Bcl-2
Bcl-2 + Bim$_S$
Bcl-2 + Bim$_L$
Bcl-2 + Bim$_{EL}$
cell number
anti-EE fluorescence intensity


B
- + + + + Bcl-2
- - Bim$_S$ Bim$_L$ Bim$_{EL}$ EE-tagged protein
Mr (kD)
32.5
27.5
18.5
IP : anti Bcl-2
BLOT: anti EE

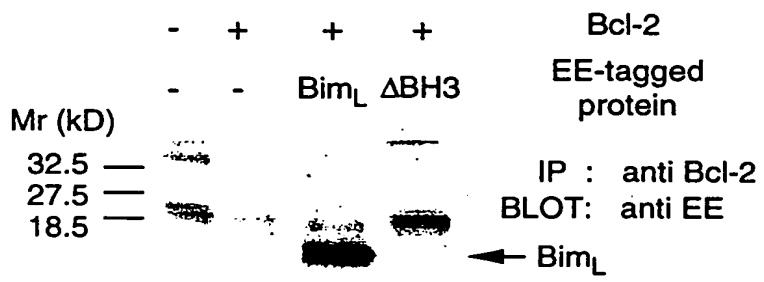

C
IL-3 deprivation
γ-irradiation
% viable cells
100
10
1
.2
0
2
4
6
8
days
parental cells
Bcl-2
Bcl-2 + Bim$_S$
Bcl-2 + Bim$_L$
Bcl-2 + Bim$_{EL}$


A
- + + + + EE Bim$_L$
wt wt mt 1 mt 7 mt 15 Bcl-x$_L$
Mr (kD)
49.5
32.5
27.5
18.5
IP: anti-EE
Bcl-x$_L$
Bim$_L$
B
- + + + + EE Bim$_L$
- Bcl-x$_L$ Bcl-w E1B19K BHRF-1 FLAG-tagged protein
Mr (kD)
49.5
32.5
27.5
IP : anti EE
BLOT: anti FLAG
32.5
27.5
18.5
BLOT: anti FLAG
27.5
18.5
BLOT: anti EE
C
% viable transfected cells
100
80
60
40
20
10
- bim$_L$
vector vector bcl-x$_L$ bcl-x$_L$ mt 1 bcl-x$_L$ mt 7 bcl-x$_L$ mt 15 other DNA
D
100
80
60
40
20
10
bim$_L$
Bcl-w E1B19K other DNA

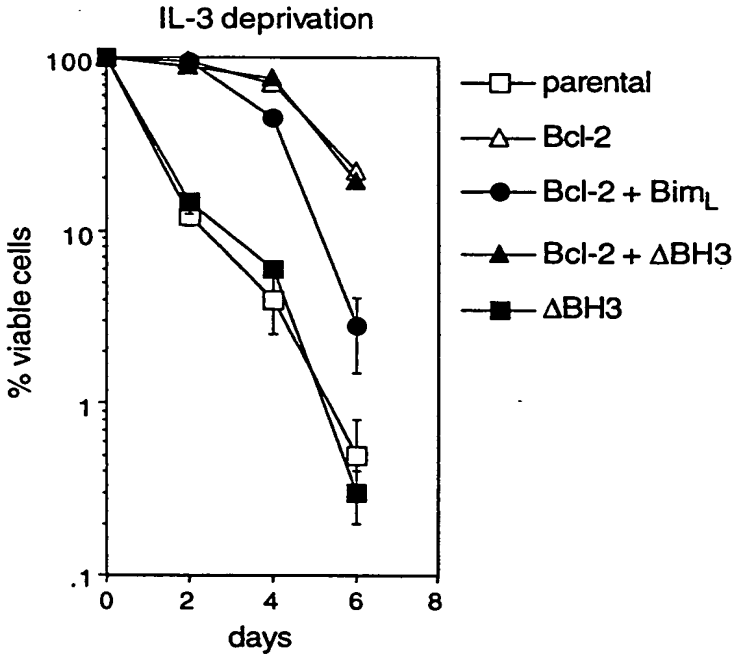
A


ΔBH3
Bcl-2 + Bim$_L$
Bcl-2 + ΔBH3
Bcl-2
cell number
anti-EE fluorescence intensity

B


- + + +
- - Bim$_L$ ΔBH3
Bcl-2
EE-tagged protein
Mr (kD)
32.5
27.5
18.5
IP : anti Bcl-2
BLOT: anti EE
Bim$_L$

C


IL-3 deprivation
100
10
1
.1
% viable cells
0
2
4
6
8
days
parental
Bcl-2
Bcl-2 + Bim$_L$
Bcl-2 + ΔBH3
ΔBH3

A

Bcl-2	92	VVHLTLR QAGDDFSRRY R	109
Bcl-x_L	85	AVKQALR EAGDEFELRY R	102
Bcl-w	41	PLHQAMR AAGDEFETRF R	58
Mcl-1	208	KALETLR RVGDGVQRNHET	227
Ced-9	53	RQASTRRMSIIGDEIDGKIND	71
Bax	58	KLSECLK RIIGDELDSN ME	75
Bak	73	QVGRQLA IIIGDDINRRY D	90
Bad	109	RYGRELR RMSDEFVDSF K	126
Bik	56	ALALRLA CIIGDEMDVS LR	73
Bid	85	NIARHLA QVGDSMDRS IP	102
Hrk	32	LTAARLK ALGDELHQR TM	49
Bim	147	WIAQELR RIIGDEFNAYYA R	165
Consensus		LR GDE	

B

Bim	147	MRPEIWIAQELRRIGDEFNA	165
Ced-4	281	QEETIRWAQELRLRCLVTRD	298